Université de Sherbrooke

Identification and isolation of multipotent stromal cells from human skeletal muscle

Par
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Programme de Pharmacologie

Mémoire présenté à la Faculté de médecine et des sciences de la santé
en vue de l'obtention du grade de Maître ès Sciences (M.Sc.)
en Pharmacologie

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RÉSUMÉ

IDENTIFICATION ET ISOLEMENT DE CELLULES STROMALES MULTIPOTENTES DU MUSCLE SQUELETTIQUE HUMAIN

Par
Jennifer Downey
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Mémoire présenté à la Faculté de médecine et des sciences de la santé en vue de l'obtention du diplôme de maître ès sciences (M.Sc.) en Pharmacologie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Le muscle squelettique humain est une source essentielle de cellules progénitrices ayant plusieurs applications thérapeutiques potentielles. Les cellules stromales mésenchymateuses du muscle squelettique humain (hmrMSCs) semblent être impliquées dans des pathologies telles l'ossification hétérotopique, la dégénérescence graisseuse et la fibrose. L'identification de la population cellulaire à l'origine de ces pathologies permettrait de mieux comprendre les mécanismes derrières celles-ci et aiderait à la création de traitements plus efficaces. Nous avons d'abord mis au point une méthode d'isolement et déterminer des conditions de culture pour la prolifération et le maintien en culture de la fraction cellulaire adhérente dérivée du muscle squelettique humain. Par le biais de la cytométrie en flux et des marqueurs connus des cellules stromales mésenchymateuses (MSC), nous avons pu enrichir les cellules stromales multipotentes. Le potentiel ostéogénique, adipogénique et chondrogénique des populations cellulaires enrichies a été évalué par des essais de différenciation. La sous-population de cellules $CD73^{+}CD105^{+}CD90^{-}$ a montré une multipotence robuste sur les trois lignées étudiées. Des essais de différenciation clonale ont confirmés que les trois lignées obtenues proviennent tous d'un progéniteur multipotent commun. De plus, cette sous-population cellulaire avait la capacité de se différencier en cellule de gras brun, démontrée par une expression élevée d'UCP1 au niveau génique et protéique suivant une stimulation continue avec le rosiglitazone (ROS). Ce résultat suggère que cette sous-population cellulaire pourrait également représenter un modèle pour l'adipogenèse vers le gras brun. La méthode d'enrichissement présentée représente une nouvelle technique afin d'obtenir des hmrMSCs. Elle semble prometteuse pour de futures applications cliniques employant ces cellules, étant donné qu'elles sont amplifiées dans un milieu défini permettant une reproductibilité inter-laboratoire. De plus, les marqueurs de phénotype choisis pour l'enrichissement par cytométrie en flux sont bien conservés entre individus, limitant la variabilité inter-donneur.

Mots clés : Cellules stromales mésenchymateuses, Cytométrie en flux, Gras brun, Maladies dégénératives, Multipotence, Muscle squelettique humain

SUMMARY

IDENTIFICATION AND ISOLATION OF MULTIPOTENT STROMAL CELLS FROM HUMAN SKELETAL MUSCLE

By
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Thesis presented to the Faculty of medicine and health sciences for the completion of the Master's degree diploma [Maitre ès Sciences (M.Sc.)] in Pharmacology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Human skeletal muscle is an essential source of various cellular progenitors with potential therapeutic perspectives. Muscle-resident mesenchymal stromal cells (mrMSCs) are thought to be involved in the development of several regenerative disorders such as fatty degeneration, heterotopic ossification and fibrosis. Identifying the cell population responsible for these pathologies will help better understand the underlying mechanisms and lead to more efficient treatment. We first developed an isolation method and culture conditions for the proliferation and maintenance of the adherent fraction of human skeletal muscle derived cells. To further enrich the cell population as multipotent progenitors, we used fluorescent-activated cell sorting (FACS) and known mesenchymal stromal cell (MSC) markers. The enriched cell populations obtained were tested for their multipotent capabilities towards the osteogenic, adipogenic and chondrogenic lineages. The $CD73^{+}CD105^{+}CD90^{-}$ subset of human skeletal muscle adherent cells displayed robust multipotence to all three lineages under the appropriate differentiation conditions. Clonal differentiation assays confirmed that all three lineages stem from a single multipotent progenitor. Furthermore, this cell subset was able to differentiate into brown adipocyte-like cells, expressing UCP1 at the RNA and protein levels following prolonged stimulation with rosiglitazone (ROS). This result suggests that this cell subset could also represent a human cell model for brown adipogenesis. The cell isolation and enrichment method presented in this thesis represent a novel technique to obtain human mrMSCs. This method holds great promise for future clinical applications with the enriched cell populations since they are expanded in a defined medium, which supports inter-laboratory reproducibility. Furthermore, the phenotypic markers chosen for the FACS isolation are well conserved amongst donors in the proposed conditions, limiting donor-to-donor variability.

Keywords: Brown adipogenesis, Fluorescent-activated cell sorting, Human skeletal muscle, Mesenchymal stromal cells, Multipotency, Regenerative disorders

To my family,
who has always encouraged
me to be curious

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LIST OF ABBREVIATIONS

Abs	Absorbance
ACL	Anterior cruciate ligament
ADIPOQ	Adiponectine
AlkP	alkaline phosphatase
ASC	Adipose stromal cell
BAT	Brown adipose tissue
BMP	Bone morphogenetic protein
CFU-F	Colony-forming unit fibroblast
CHUS	Centre hospitalier universitaire de Sherbrooke
CIDEA	Cell death-inducing DFFA-like effector A
cMEC	clonal myoendothelial cells
Col2a1	Type II collagen
COX	Cytochrome c oxidase
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
FAB4	Fatty acid binding protein 4
FACS	Fluorescence-activated cell sorting
FAP	Fibro/adipogenic progenitors
FBS	Fetal bovine serum
FOP	Fibrodysplasia ossificans progressiva
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPI	Glycosyl-phosphatidylinositol
GS	Glycine-serine
hMAD	human multipotent adipose derived stem cells
hmrSC	Human muscle resident stromal cell
HO	Heterotopic ossification

HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
HSMM	Human skeletal muscle myoblast
Ig	Immunoglobulin
MDSC	Muscle-derived stem cells
MEC	Myoendothelial cells
MHC	Myosin heavy chain
MMP	Matrix metalloproteinase
MPC	Myogenic progenitor cell
MPC	Mesenchymal progenitor cell
MRF	Myogenic related factor
mrSC	muscle-resident stromal cell
MSC	Mesenchymal stromal cell
NHDF	Normal human dermal fibroblast
NSAID	Nonsteroidal anti-inflammatory drugs
OC	Osteocalcin
Osx	Osterix
PDGFRα	Platelet-derived growth factor receptor alpha
PGC1α	Peroxisome proliferator activated receptor gamma coactivator 1α
PPARγ	Peroxisome proliferator activated receptor-γ
PRDM16	PRD1-BF-1-RIZ1 homologous domain containing protein-16
PRE	PPARγ-response element
ROS	Rosiglitazone
Runx2	Runt-related transcription factor 2
SP	Side population
SSC	Side scatter
TBP	TATA-box binding protein
TGFβ1	Transforming growth factor-β1
THA	Total hip arthroplasty

TIMP	Tissue inhibitor of metalloproteinase
UCP1	Uncoupling protein 1
WAT	White adipose tissue
αSMA	Alpha-smooth muscle actin

FOREWARD

Due to its marked regenerative capacity, skeletal muscle is one of the most studied tissues in regenerative biology. Despite significant advances in understanding the process of muscle tissue regeneration after injury or disease, the identity of the progenitor cells involved has been a matter of considerable debate, particularly with respect to regenerative disorders such as fibro/adipogenic degeneration and heterotopic ossification (HO).

Mesenchymal stromal cells (MSCs) are a population of multipotent adult progenitor cells with many properties that make them attractive for use in the field of regenerative medicine (Aldahmash *et al.* 2012). These cells are inherently plastic, enabling them to differentiate along different lineages. They also appear to exhibit a number of trophic properties that promote regeneration in the surrounding tissue (Caplan 2009). Furthermore, MSCs can be harvested from a variety of adult tissues (da Silva Meirelles *et al.* 2006), and of particular interest in this thesis from skeletal muscle (Huard 2008).

In spite of the potentially far-reaching promise of MSCs in many aspects of regenerative medicine, approaches using these cells are limited by the availability of a suitable MSC population in a clinical setting. Another obstacle arises from the identification of MSC populations due to a lack of standardization with respect to methods for their isolation and expansion.

This thesis focuses on the enrichment and characterization of a muscle resident stromal progenitor cell population from adult human skeletal muscle.

1. INTRODUCTION

1.1. Skeletal muscle tissue

Skeletal muscle is the largest tissue mass in the body, making up approximately 40% of the total body weight (Huard *et al.* 2002). As a form of striated muscle tissue, its main function is to perform locomotor activity under the voluntary control of the somatic nervous system. In addition, it is documented to have an important role in metabolism and its perturbation may contribute to metabolic diseases such as metabolic syndrome (Wells *et al.* 2008).

1.1.1. Structure of skeletal muscle tissue

As the name suggests, most skeletal muscles are attached to the bones by tendons. Although skeletal muscle is composed primarily of contractile material, notably muscle fibers, it is a composite tissue of blood vessels, connective tissue, and nerves. Several layers of connective tissue encompass the muscle. The outer layer, the epimysium, groups several fascicles that surround the entire muscle and extend into the muscles' tendons. Each fascicle, made up of parallel myofibers, also called muscle fibers, is enclosed within the perimysium. The endomysium, also referred to as the basal lamina, is the connective tissue that surrounds each individual myofiber **(Fig. 1.1)**. The later separates myofibers from the surrounding connective tissue through which blood vessels run to provide nutrition, nerves to induce fiber contraction, and muscle resident stromal cells which maintain tissue integrity (Gillies and Lieber 2011).

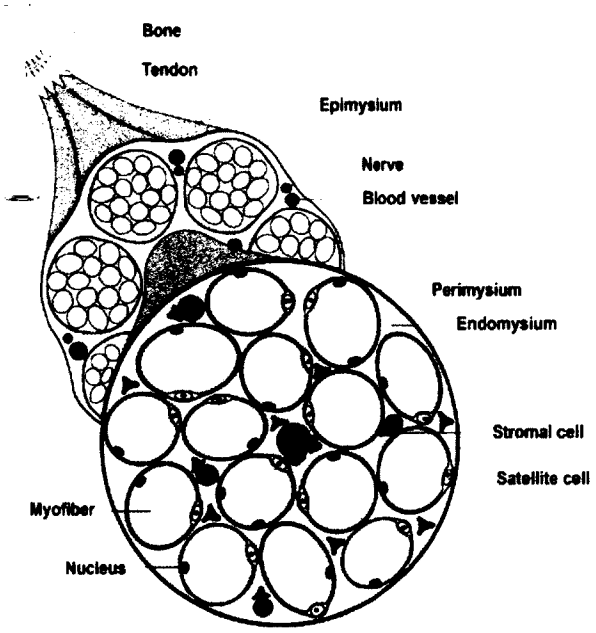


Figure 0.1: Skeletal muscle structure and organization

Illustration showing the organization of the skeletal muscle. Each myofiber is enclosed in a basal lamina and arranged in functional units called fascicles encompassed within three connective tissue sheaths: the endomysium (surrounding myofibers), the perimysium (surrounding fascicles) and the epimysium (surrounding the muscle). The myofibers are also surrounded by an important vascular network which is represented by the pink blood vessels. This network is extensive however for clarity only a few vessels are shown in this illustration. The stromal cells (in blue) are suggested to be perivascular cells, which is illustrated by their proximity to the blood vessels. Adapted from (Trensz *et al.*, unpublished)

The muscle fiber, is the functional cell of skeletal muscle tissue. It is a syncytium originating from the fusion of hundreds of myogenic progenitor cells (MPCs). Each myofiber has hundreds of nuclei in its periphery and is composed of parallel myofibrils, which are composed of sarcomeres, the contractile unit of the muscle. It is the banding pattern of sarcomeres, an arrangement of thick myosin and thin actin filaments that gives skeletal muscle its "striated" appearance **(Fig. 1.2)**. It is this high level of micro- and macro-organization that enables optimal contraction, which allows the muscle to fulfill its primary function.

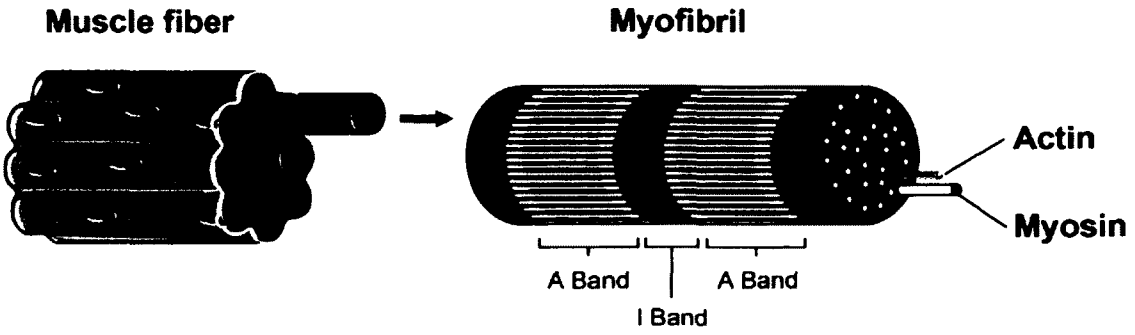


Figure 0.2: Internal structure of a muscle fiber

Muscle fibers are made up of myofibrils, which contain actin and myosin filaments. Muscle fiber organization with A bands and I bands gives skeletal muscle its striated appearance. Adapted from Servier medical art (Servier 2010).

1.1.2. Satellite cells

The highly effective regenerative capacity of adult skeletal muscle is attributed to a population of mononucleated myogenic stem cells called satellite cells (Biressi and Rando 2010; Yin *et al.* 2013). These cells were first described by their anatomical localization beneath the basal lamina surrounding the plasma membrane of the myofiber (sarcolemma) (Mauro 1961) **(Fig. 1.3)**. This location provides a unique microenvironment that maintains satellite cells in a quiescent state under normal conditions. However, following tissue damage, environmental cues activate these cells. Once activated, satellite cells proliferate before fusing with pre-existing myofibers, or they form entirely new myofibers as they undergo a terminal differentiation process (Charge and Rudnicki 2004). To simplify nomenclature, only quiescent cells are called satellite cells. Once these cells are activated, they are called myogenic precursor cells (MPCs) or myoblasts *in vitro*.

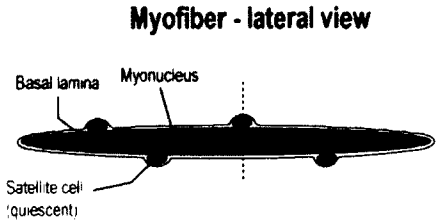


Figure 0.3: The myofiber and its satellite cells

The muscle fiber is a multinucleated cell originating from the fusion of myogenic progenitor cells (MPCs). Satellite cells are found between the plasma membrane of the myofiber and the basal lamina which surrounds it. Normally quiescent, satellite cells are activated following muscle damage in order to regenerate the myofibers. (Grenier *et al.* unpublished).

Other than location, the identification of satellite cells relies on specific biomarkers. In adult skeletal muscle, the paired domain transcription factor Pax7 is the canonical biomarker for satellite cells (Seale *et al.* 2000; Morrison *et al.* 2006; Yablonka-Reuveni 2011). It is specifically expressed in all quiescent and proliferating satellite cells across multiple species, including humans (McLoon and Wirtschafter 2003). Although skeletal muscle normally has a slow turnover rate, following an important trauma, muscle tissue is capable of a rapid and complete regeneration (Charge and Rudnicki 2004).

1.2. Skeletal muscle regeneration

Following damage, as is the case after intensive exercise or trauma, skeletal muscle has the remarkable ability to initiate a rapid and extensive repair process. It is one of the few adult tissues that possesses this regenerative capacity. The regeneration of mature skeletal muscle is a complex process composed of a degenerative and a regenerative phase involving satellite cells, MPCs, immune cells, and the remodeling of the connective tissue by myofibroblasts (Charge and Rudnicki 2004). The damage triggers changes in the satellite cells microenvironment, leading to their activation. Satellite cells respond to these changes by re-entering the cell cycle to both self-renew and to generate MPCs, which is a process of asymmetric division, that will eventually undergo terminal differentiation and fuse with myofibers to repair damage (Wang and Rudnicki 2012). The process of myogenic repair by satellite cells and MPCs is carefully orchestrated by the expression of specific myogenic transcription factors **(see section 1.2.2)**.

1.2.1. Degenerative phase

Muscle degeneration begins with necrosis of damaged muscle fibers **(Fig. 1.4)**. This event is initiated by increased myofiber permeability, which is reflected by increased plasma levels of intracellular myofiber proteins. Myofiber necrosis also activates the complement cascade and induces inflammatory responses (Orimo *et al.* 1991) resulting into the recruitment of circulating leukocytes at damage sites (Gute *et al.* 1998; Figarella-Branger *et al.* 2003). Neutrophils are the first inflammatory cells to infiltrate the damaged muscle (Fielding *et al.* 1993), followed by macrophages which become the predominant inflammatory cells (Orimo *et al.* 1991). The presence of inflammatory cells is essential for the phagocytosis of tissue debris, however it is also responsible for the production of cytokines which are important for the initiation of the second phase of the repair process (Bischoff 1997).

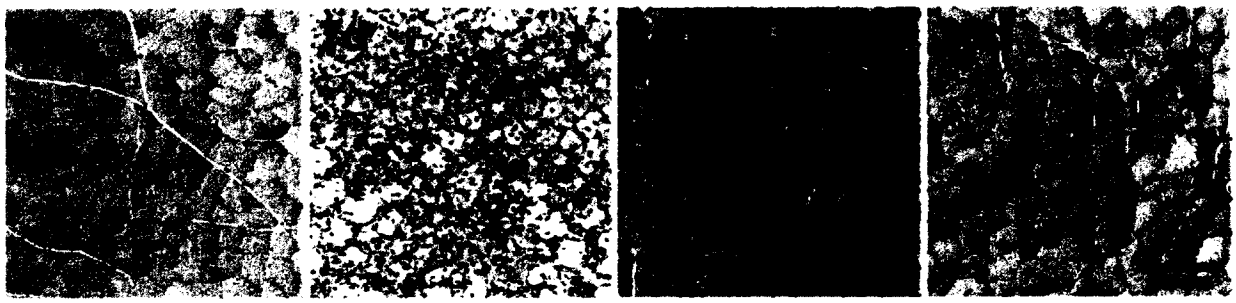


Figure 0.4: Muscle tissue regeneration

Mouse skeletal muscle tissue regeneration in a transverse section following a hematoxylin and eosin staining. The images represent muscles at days 0, 7, 14 and 28 following muscle damage (cardiotoxin injection). Muscle regeneration is characterized by muscle fiber degeneration accompanied by inflammatory cell infiltration (7d). This is followed by muscle fiber repair by MPCs which fuse together to form new myofibers characterized by a small diameter and the presence of central nuclei (arrowhead) (14d). At day 28, most of the myofibers are mature and have regained their initial dimension. Their nuclei have also migrated back to the periphery from the center. (Grenier *et al.*, unpublished).

1.2.2. Regenerative phase

The second phase of the repair process concerns muscle fiber regeneration. There are markers that distinguish satellite cells from proliferative and mature MPCs, such as transcription factors from the myogenic related factors (MRFs) family including MyoD,

Myf5 and myogenin. As mentioned earlier, quiescent satellite cells are characterized by their expression of Pax7, and Myf5 to a lesser extent. The latter is an important factor in the management of the satellite cell pool contributing to muscle homeostasis (Kuang *et al.* 2007). Interestingly, the deletion of Myf5 induces muscle fiber hypertrophy and Myf5$^{-/-}$ MPC proliferation (Gayraud-Morel *et al.* 2007; Ustanina *et al.* 2007). MyoD is the first sign of satellite cell activation and its expression rapidly increases following muscle damage. Mice deficient for MyoD have less muscle mass and present delayed myogenic differentiation (Yablonka-Reuveni *et al.* 1999; White *et al.* 2000). Also following muscle damage, MyoD deficient mice show an accumulation of MPCs in the site of the damage incapable of differentiating and fusing (Megeney *et al.* 1996). While MyoD and Myf5 expression encourages satellite cells to differentiate towards the myogenic lineage **(Fig. 1.5)**.

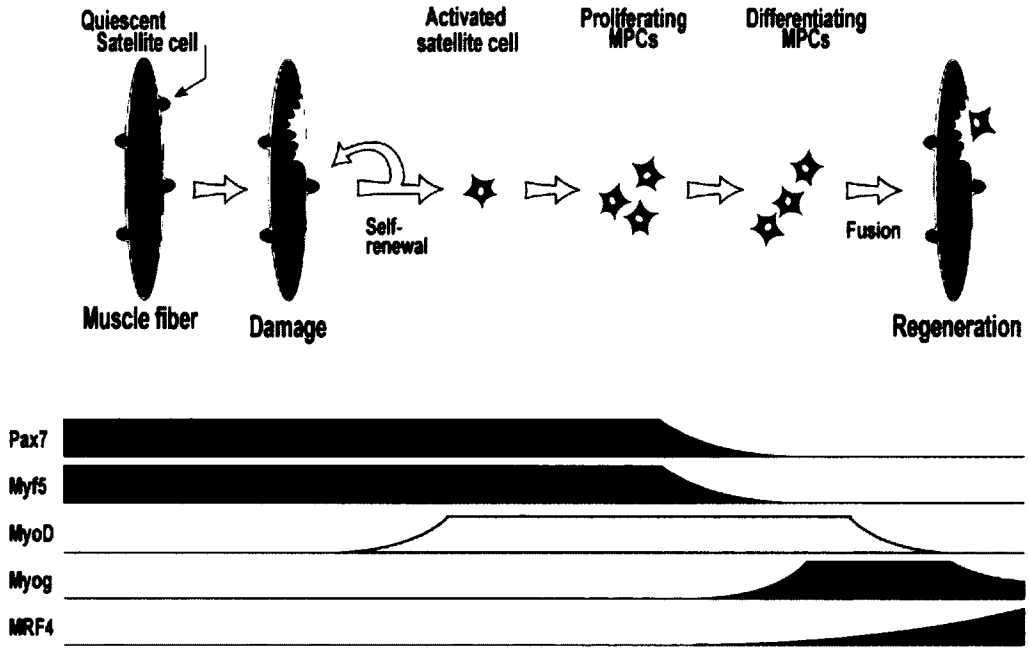


Figure 0.5: Key factors involved in myogenic differentiation

Muscle damage induces the activation, proliferation and differentiation of satellite cells in order to repair damaged muscle fibers or create new ones. These stages are regulated by specific transcription factors. Pax7 and Myf5 are expressed by quiescent, activated and proliferating satellite cells. MyoD is specific for proliferating MPCs and those beginning differentiation. During maturation and fusion MPCs express myogenin and MRF4. These transcription factors can be used as markers to identify the differentiation stage (Trensz *et al.*, unpublished).

Following the MPCs proliferation phase, myogenin is expressed during terminal differentiation and fusion. Myogenin expression enables MPCs to exit the cell cycle, through p21 expression. Unlike the other transcription factors discussed, the loss of myogenin leads to severe skeletal muscle deficiencies, highlighting its essential role in the differentiation of MPCs during embryogenesis (Venuti *et al.* 1995). Myogenin is linked to muscle fiber fusion and maturation. The differentiation process is then completed by the activation of muscle specific proteins such as myosin heavy chain (MHC), the motor protein of muscles thick filaments, leading to functional muscle fibers. Histologically, newly formed myofibers are characterized by central nuclei (Charge and Rudnicki 2004). Once MPC fusion is complete, newly formed myofibers increase in size due to functional protein synthesis, and myonuclei move to the periphery of the muscle fiber **(Fig. 1.4)**.

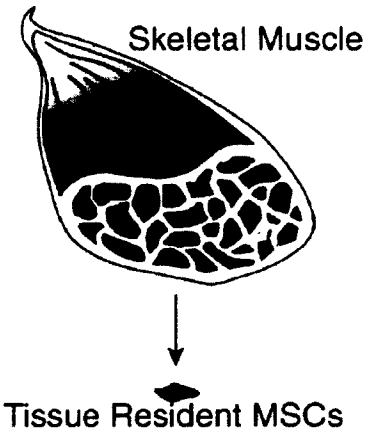
After injury, tissue regeneration involves not only the replenishment or replacement of parenchymal cells but also that of supporting structures including blood vessels, nerves, connective tissue and stromal cells. Little is known about how this network of associated cells, notably MSCs, coordinates myofiber growth, homeostasis and repair in skeletal muscle.

1.3. Skeletal muscle regenerative disorders

Although tissue regeneration is ensured by tissue-specific stem cells, efficient tissue repair requires activation of the surrounding stromal microenvironment, mainly α-smooth muscle actin (αSMA) positive myofibroblasts, to produce growth and survival factors, pro-inflammatory chemokines and components of the extracellular matrix, such as collagens. However, inappropriate activation of the stromal compartment can lead to persistence of αSMA^{+} collagen-overproducing cells, extracellular matrix (ECM) accumulation and overgrowth of fibrous and/or adipogenic tissue, thereby compromising tissue recovery and impairing function (Wynn 2008). Therefore, tissue repair culminates in either complete restoration of tissue integrity, defined as regeneration, or in a process that leads to the generation of stromal structures that replace functional tissue, which I will entitle 'regenerative disorders' in this thesis. The three most commonly occurring outcomes of

regenerative disorders are fibrosis, fatty degeneration and heterotopic ossification (HO) **(Fig 1.6)**.

The accumulation of these mature cell types in tissues that have failed to properly regenerate suggests that alterations in the function of muscle resident mesenchymal stromal cells (mrMSCs) may represent a common thread underlying regenerative disorders. Unlike MPCs, the identity of the stromal progenitor responsible for mesenchymal lineages in skeletal muscle remains unclear.



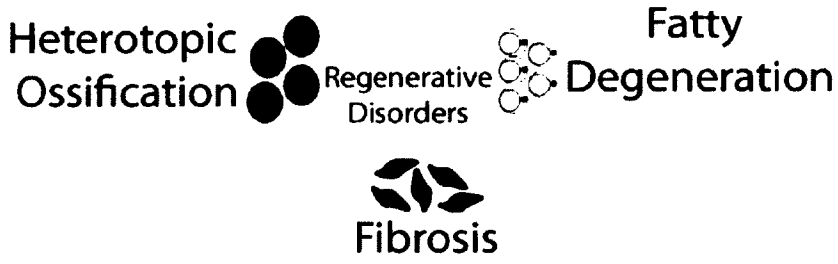


Figure 0.6: MSCs and skeletal muscle regenerative disorders

During injury or disease skeletal mrMSCs can expand and provide trophic support for regeneration and/or differentiate to produce fibrosis, fatty degeneration or HO, or a combination of these (Adapted from Pretheeban *et al.* 2012).

1.3.1. Fibrosis and fatty degeneration

After chronic injury, muscle is often replaced by a mix of fibrous tissue and white adipocytes in a process termed fatty degeneration. This fibro/adipogenic infiltration compromises muscle function and alters the tissue microenvironment, potentially limiting the success of regenerative approaches.

Pathophysiologic fibrosis is a defining characteristic in most reparative disorders involving chronic inflammation (Porter *et al.* 2002). It is characterized by excessive accumulation of ECM in which collagen type I is the major component. It is the end result of a cascade of events proceeding from tissue injury via inflammation, and resulting in permanent scar formation. The accumulated ECM replaces normal parenchymal tissue and can affect tissues and organ systems, resulting in anatomical anomalies as well as reduced functional capacities. In skeletal muscle, fibrosis is most often associated with the muscular dystrophies. As well as the muscular dystrophies, aging is associated with loss of skeletal-muscle mass and function with concomitant fibrosis and ECM deposition (Mann *et al.* 2011).

The accumulation of fat in damaged tissue leading to "fatty degeneration" is another common regenerativedisorder (Moyer and Wagner 2011). Usually, fat is found in newly formed adipocytes infiltrating the tissue, most often associated with concurrent fibrotic matrix deposition, and associated with injuries and defective repair processes (Wallace and McNally 2009). The appearance of both fat accumulation and fibrosis has been shown in macrophage-suppressed mice (Warren *et al.* 2005; Segawa *et al.* 2008). Fat accumulation and fibrosis are also observed in mice lacking Myf5, a myogenic transcription factor (Gayraud-Morel *et al.* 2007). Furthermore, it is widely known that an increase in fatty and fibrous connective tissue is a hallmark of advanced Duchenne muscular dystrophy (DMD), which is caused by a mutation of the dystrophin gene (Carpenter and Karpati 2001).

The poorly defined fibroblast or myofibroblast, the effector cell component of connective tissue, is generally thought to be responsible for producing excessive collagen and other ECM proteins (Hinz and Gabbiani 2010). However, the origin of fibrosis in

skeletal muscle has not been elucidated (Mann *et al.* 2011). It has been suggested that both adipogenic and fibrogenic cells originate from myogenic cells through alternative lineage choices dictated by a pathological environment (Li *et al.* 2004; Shefer *et al.* 2004; Brack *et al.* 2007). As an example, muscle side population (SP) cells isolated from dystrophic or cardiotoxin-injured muscle, which normally contribute to myogenesis and muscle regeneration, failed to undergo myogenesis and instead gave rise to fibroblasts and adipocytes (Penton *et al.* 2013), suggesting that muscle damage affects lineage choices of muscle SP cells for a possible role in fibrosis and fat deposition. The fate of these progenitors is therefore heavily dependent on their microenvironment. This local microenvironment dictates whether these cells provide trophic support to satellite cells, the endogenous myogenic stem cells, to yield complete regeneration of injured muscle or whether they generate the components of the fibro-fatty tissue infiltrates often found in degenerating muscle tissue. On the other hand, a population of skeletal muscle resident mesenchymal progenitors expressing platelet-derived growth factor receptor alpha ($PDGFR\alpha^+$) has been shown to contribute to ectopic fat formation and fibrosis in mouse skeletal muscle under pathogenic conditions (Joe *et al.* 2010; Uezumi *et al.* 2011). Also, chronic activation of $PDGFR\alpha^+$ cells leads to widespread organ fibrosis in mice (Olson and Soriano 2009).

1.3.2. Heterotopic ossification

HO is another regenerative complication involving the formation of cartilage and bone outside of the normal skeleton, within soft tissue such as muscle. Overall, it decreases range of motion and can cause partial or complete joint ankylosis for which surgical intervention is required. Usually, the development of HO is either genetic (Shore and Kaplan 2010) or acquired following a traumatic injury. (Vanden Bossche and Vanderstraeten 2005; Potter *et al.* 2007).

1.3.2.1. Fibrodysplasia ossificans progressiva

The most severe form of HO is manifested in the rare, autosomal dominant genetic disorder, *fibrodysplasia ossificans progressiva* (FOP), in which heterotopic bone forms

progressively throughout the life of the individual, resulting in devastating effects on health, life expectancy, and quality of life (Kaplan *et al.* 2004; Vanden Bossche and Vanderstraeten 2005). It was discovered that FOP results from the missense mutations in the highly conserved glycine-serine (GS) regulatory domain of the bone-morphogenetic protein (BMP) Type I receptor 1A/activin-like kinase-2 (ACVR1/Alk2) (Shore *et al.* 2006; Fukuda *et al.* 2009), which renders the BMP signalling pathway hypersensitive or independent of BMP ligands (Shore *et al.* 2006; Billings *et al.* 2008; Fukuda *et al.* 2009). Injury is also a trigger for HO in FOP; however, in this genetically susceptible background, even mild soft tissue trauma can cause pronounced heterotopic skeletogenesis (Kaplan *et al.* 2008).

1.3.2.2. Traumatic heterotopic ossification

Traumatic HO is the most common type of HO, observed following fractures, burns, surgical traumas, especially total hip arthroplasty (THA) (Nilsson and Persson 1999). For example, HO is diagnosed on average in 53% of patients after THA. Of this number, 10% of patients suffer from severe HO with pain in the area of the operated joint combined with a decrease in the range of motion, leading to functional impairment (Brooker *et al.* 1973; Thomas 1992). Not to mention, HO is also prevalent in patients with severe extremity wounds following a polytraumatic injury, in fact in these cases the incidence of HO increases by 57% (Potter *et al.* 2007). The incidence of HO is also increased following central nervous system injuries (Newman *et al.* 1987; van Kuijk *et al.* 2002; Sakellariou *et al.* 2012). The range of these conditions suggests that factors released by distant tissues such as the brain may have an osteoinductive effect on soft tissues either directly or by stimulating local cells to produce osteogenic growth factors (Gautschi *et al.* 2009).

1.3.2.3. HO pathogenesis

Although the pathogenesis of HO is not fully understood, it is generally agreed that an inciting event, such as a trauma induces local inflammation (McCarthy and Sundaram 2005). This is followed by the recruitment of surrounding osteogenic progenitor cells that differentiate into chondrocytes, undergo hypertrophy and are replaced by endochondrale

bone (Shore and Kaplan 2010). Therefore, HO is thought to result from the inappropriate differentiation of tissue resident osteogenic progenitor cells that are induced by a pathological imbalance of local or systemic factors. However, the precise cell origin and environmental cues leading to HO have not been fully elucidated. Since the most common cases of HO are in muscle and soft tissues (Nilsson and Persson 1999), it is important to understand the osteogenic properties of cells resident in skeletal muscle.

Among local tissue resident populations, satellite cells have received considerable attention as a possible cell-of-origin, primarily because of their muscle-restricted distribution and their capacity for BMP-dependent osteogenic differentiation in culture (Katagiri *et al.* 1994; Wada *et al.* 2002). Cre/lox-based lineage analysis in the mouse, however, demonstrated that satellite cells *in vivo* do not appreciably contribute to HO lesions (Kan *et al.* 2009; Lounev *et al.* 2009).

1.4. Mesenchymal stromal cells

Friedenstein *et al.* was the first group to isolate multi-potential stromal precursor cells from the bone marrow (BM-MSCs). They described adherent, spindle-shaped, colony-forming unit fibroblasts (CFU-Fs) capable of differentiating into adipocytes, chondrocytes and osteocytes both *in vitro* and after transfer *in vivo* (Friedenstein *et al.* 1974) **(Fig. 1.7)**. It was subsequently demonstrated that these cells were also capable of multi-lineage differentiation at the clonal level (Pittenger *et al.* 1999). A clonal differentiation assay is important since it supports the hypothesis that the differentiation potential belongs to a single multipotent progenitor rather than unipotent subpopulations.

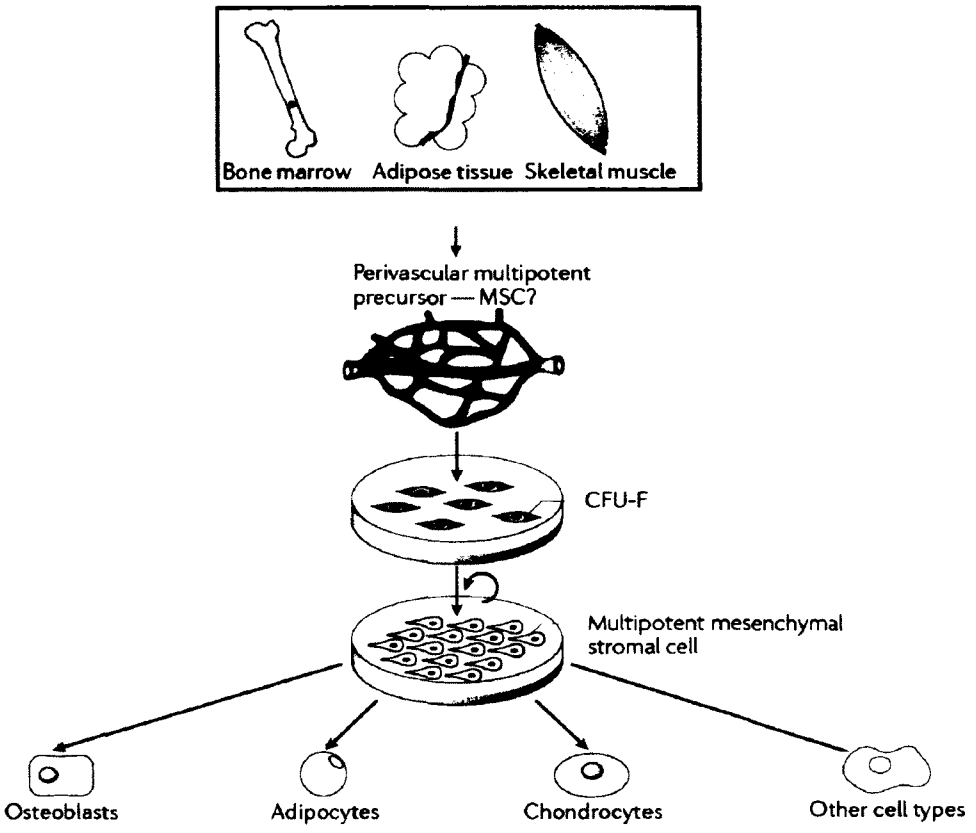


Figure 0.7: Multipotent mesenchymal stromal cell characteristics

The plastic-adherent cellular fraction of many organs contains stromal progenitor cells that can give rise to colonies of fibroblastic morphology. This cellular subset, known as CFU-Fs, can give rise to a proposed multipotent progenitor cell population, most probably heterogeneous in nature that resides in the perivascular location. Cultured under the appropriate conditions these cells can be expanded for multiple passages *in vitro* without losing their multipotent capabilities. The hallmark that defines MSCs is their ability to differentiate towards osteogenic, adipogenic and chondrogenic cell fates under the appropriate stimuli. Differentiation into other non-mesenchymal mature cell types remains a matter of debate (adapted from Silberstein *et al.* 2011).

The regenerative benefit of MSCs does not appear to correlate solely with their ability to differentiate into the diseased tissue type (Caplan and Dennis 2006). MSCs exert trophic functions in the environment of damaged tissues that promote endogenous wound healing mechanisms (Caplan 2009), such as promoting angiogenesis (Tang *et al.* 2004; Martens *et al.* 2006), reducing fibrosis (Li *et al.* 2008; Mias *et al.* 2009) and modulating inflammation (Le Blanc *et al.* 2003; Kode *et al.* 2009). It has since been shown that MSCs

also secrete trophic (pro-growth and pro-survival) factors that augment the endogenous regeneration process (Tang *et al.* 2004; Lozito *et al.* 2009).

Therefore, multipotent mesenchymal stromal cells can be defined as a heterogeneous population of cells that proliferate *in vitro* as plastic-adherent cells, have fibroblast-like morphology, form colonies *in vitro* and can differentiate into bone, cartilage and fat cells (Horwitz *et al.* 2005). Since their original description, MSCs categorized based on trilineage (osteoblast, adipocyte and chondrocyte) potential *in vitro* have been isolated from the adherent fraction of many adult tissues in multiple species (da Silva Meirelles *et al.* 2006).

1.4.1. Tissue sources of MSCs

In addition to bone marrow aspirate, MSCs have been isolated from a variety of other adult tissues, including adipose tissue (Gimble and Guilak 2003; Meliga *et al.* 2007), skin (Toma *et al.* 2001), the marrow space of long bones (Kuo and Tuan 2003; Tuli *et al.* 2003), trabecular bone (Tuli *et al.* 2003; Song *et al.* 2005), synovial membranes (De Bari *et al.* 2001), and periosteum (Nakahara *et al.* 1991; Choi *et al.* 2008) to name a few. However the limited availability of these tissues may limit their clinical potential. Similarly, MSCs have been harvested from tissues that are lost as a result of development, such as the umbilical cord (Lee *et al.* 2004; Baksh *et al.* 2007) and umbilical cord blood/Wharton's Jelly (Cetrulo 2006; Troyer and Weiss 2008).

Of particular interest, there have also been several reports of harvesting MSC-like cells from murine and human adult skeletal muscle. Human muscle tissue used to harvest cells was obtained from healthy muscle tissue biopsies (Zheng *et al.* 2007), surgical waste tissue from orthopaedic reconstructions, or surgically debrided muscle tissue following orthopaedic trauma (Nesti *et al.* 2008). Given that these cells can be obtained from surgical waste tissue or from minimally invasive biopsy procedures, there is growing evidence that skeletal muscle may be an important clinical source of MSCs for use in therapeutic applications.

1.4.2. Identification of MSCs

Identifying MSCs *in vivo* is difficult, due to their low abundance. Consequently, the majority of work studying the properties of MSCs has been performed using cultured MSCs which are selected by adherence to tissue culture plastic, followed by differentiation assays to test their multilineage potential. This selection method makes it difficult to compare MSC populations from different species, isolation techniques, culture conditions, and donor sites.

Multipotence and adherence to tissue culture plastic are important characteristics of MSCs, yet there is no definitive, agreed-upon marker to positively identify a population that is capable of these functions (Kolf *et al.* 2007). Instead, human MSCs are identified based on the expression of a number of surface markers comprising CD105, CD90, CD73, CD71, CD44 and Stro-1 (Moroni and Fornasari 2013). Adhesion molecules like CD166, CD106, ICAM-1, and CD29 are also reported to be expressed on MSCs, while hematopoietic surface markers, such as CD45, CD34, CD14 and CD11, are not. MSCs are also characterized by a negative expression of co-stimulatory markers like CD86, CD80, and CD40, as well as specific adhesion molecules related to platelet/endothelial cells (CD31 or PECAM-1), to neural adhesion (CD56), and to leukocyte function (CD18 or LFA-1) (Moroni and Fornasari 2013).

These markers were essentially developed for cells harvested from the bone marrow. Therefore, additional tissue-specific criteria may also apply to this cell population from other tissues. In fact, these surface markers are not homogeneously expressed throughout stromal cultures and they may vary with tissue source (Al-Nbaheen *et al.* 2013). This raises the question of whether MSCs from different anatomical locations, selected by classic adherence and *in vitro* culture methods are biologically equivalent.

Interestingly, MSCs derived from various embryonic and postnatal tissues using identical culture conditions display significant differences in colony forming morphology, differentiation potential and gene expression (Lee *et al.* 2004; Panepucci *et al.* 2004; da Silva Meirelles *et al.* 2006; Kaltz *et al.* 2010). Furthermore, studies have characterized and

compared the immunophenotype of cultured adipose-derived stromal cells (ADSCs), in early and later passages, and found that the expression profile changes during culture time. It has been repeatedly shown that freshly isolated ADSCs express different surface markers than those in later passages (Mitchell *et al.* 2006; Varma *et al.* 2007). Many of the markers, particularly surface proteins, may be differentially up- or down-regulated *in vitro*, possibly influenced by culture conditions such cell confluence and medium, which may be especially true when using serum-based culture medium.

1.4.3. Cell surface markers for MSCs

Although MSCs do not express unique phenotypic markers, the International Society for Cellular Therapy proposed minimal criteria (Dominici *et al.* 2006) for defining MSCs based on their plastic adherence, phenotype and trilineage multipotence in order to help standardize their identification amongst researchers. The phenotype definition requires the expression of CD73, CD90 and CD105, together with a lack of expression of haematopoietic progenitor and endothelial cell markers (CD34), a leukocyte marker (CD45) and several others markers specific for other cell types. However, these markers were identified in studies done using mostly human bone marrow MSCs and it is still to be determined whether they apply to human muscle-derived MSCs.

1.4.3.1. CD73

CD73 is an ecto-5'-nucleotidase, which is known to be involved in bone marrow stromal interactions (Barry *et al.* 2001), mesenchymal stem cell migration (Ode *et al.* 2011), and potentially, MSC modulation of adaptive immunity (Eckle *et al.* 2007).

1.4.3.2. CD105 (endoglin)

Several groups have reported the expression of the cell surface receptor CD105 (endoglin) to be correlated with stem cell capacity within mesenchymal cells of adipose tissue and bone marrow origin (Rada *et al.* 2011). Mechanistically, CD105 has been shown to function as a TGFβ1 co-receptor (Castonguay *et al.* 2011; Zhang *et al.* 2011). CD105 has also been found to be involved in a variety of other biological processes. Studies have

linked CD105 to angiogenesis and neovascularization, the development of pre-eclampsia, scleroderma, and psoriasis (Castonguay *et al.* 2011; Holmes *et al.* 2011; Nassiri *et al.* 2011; Pohl *et al.* 2011)

1.4.3.3. CD90 (Thy-1)

CD90 (Thy-1) is a glycosyl-phosphatidylinositol (GPI)-linked membrane protein which has been shown to be associated with osteoprogenitor cells (Chen *et al.* 1999; Chan *et al.* 2009; Nakamura *et al.* 2010). CD90 is a member of the immunoglobulin (Ig) supergene family and is also expressed on the surface of thymocytes, peripheral T cells, fibroblasts, epithelial cells, neurons, and hematopoietic stem cells (Reif and Allen 1964; Blankenhorn and Douglas 1972; Williams 1982). Interestingly, CD90 expression varies with species (Dalchau and Fabre 1979; Kemshead *et al.* 1982) as well as the state of differentiation (Chen *et al.* 1999).

1.4.4. Multilineage potential of MSCs

Beyond their ability to generate osteoblasts, adipocytes and chondrocytes *in vitro*, MSCs give rise to bone and cartilage after ectopic implantation *in vivo* (Ashton *et al.* 1980; Haynesworth *et al.* 1992) and have been documented to contribute to bone regeneration in animal models of genetic bone disorders (Li *et al.* 2010).

Many studies have further reported MSC differentiation into multiple other cell types of mesodermal and non-mesodermal origin, including endothelial cells (Oswald *et al.* 2004), cardiomyocytes (Makino *et al.* 1999), hepatocytes (Snykers *et al.* 2009) and neural cells (Phinney and Prockop 2007; Arthur *et al.* 2008). Although the multipotent capabilities of MSCs are controversial, their differentiation into the osteogenic, adipogenic and chondrogenic lineages are now widely accepted and well documented.

1.4.4.1. Osteoblastic differentiation

Osteoblasts develop through a series of phases, initiated by cellular proliferation, followed by ECM deposition and matrix mineralization. This process of cell maturation can be induced *in vitro* by the addition of BMPs, such as BMP2 (Diefenderfer *et al.* 2003), or the addition of a differentiation cocktail of dexamethasone, ascorbate and β-glycerophosphate (Jaiswal *et al.* 1997). A range of transcription factors are known to be involved in the regulation of osteogenic differentiation (Marie 2008), with the two most popular being runt-related transcription factor 2 (Runx2/Cbfa1) and Osterix (Osx). Runx2 is considered the major transcription factor controlling osteoblast commitment and differentiation. It is expressed early in skeletal development and throughout osteoblast differentiation. Osx, another important transcription factor involved in osteoblast commitment, appears to act downstream of RunX2. Osx is thought to act in the regulation of numerous osteoblast genes including, osteocalcin (OC). The latter is a bone specific protein synthesized by osteoblasts and represents a good marker for osteogenic maturation (Nakamura *et al.* 2009).

1.4.4.2. Adipogenic differentiation

Adipocytes mature though a series of increasingly committed cell types, before expressing adipocyte specific markers such as fatty acid binding protein 4 (FAB4) (Samulin *et al.* 2008) and forming lipid vesicles which can be detected by oil red O staining. Peroxisome proliferator activated receptor-γ (PPARγ) is a nuclear hormone receptor, thought to be the master regulator of adipogenesis. There are two isoforms of PPARγ, PPARγ1 is ubiquitously expressed whilst PPARγ2 is restricted to adipose tissues and appears to be a more potent stimulator of adipogenesis (Mueller *et al.* 2002). PPARγ is expressed early in the differentiation of adipocytes, with forced overexpression of PPARγ inducing adipogenesis in cultured fibroblast (Tontonoz *et al.* 1994).

1.4.4.3. Chondrogenic differentiation

Chondrogenic differentiation *in vivo* requires an initial condensation of the MSCs, which is mimicked *in vitro* by culturing MSCs as micromass pellets. Chondrogenic

differentiation can then be induced by the presence of transforming growth factor-β (TGFβ) resulting in the appearance of a chondrocyte-like phenotype characterized by the up-regulation of cartilage-specific molecules such as collagen type II and IX, aggrecan, versican, biglycan and decorin (Pelttari *et al.* 2008). Chondrocyte maturation evolves though a sequence of defined steps; initially differentiating cells are termed chondroblasts and are proliferative. This stage is then followed by a hypertrophic stage, marked by the expression of collagen type-X, which is vital *in vivo* for vascular invasion, osteoblast differentiation, and bone formation. Sox9 is a high-mobility group box-containing transcription factor and is considered the master regulator of chondrogenesis. The Sox9 gene is expressed in all chondrocyte progenitors and chondrocytes, but its expression is completely turned off in hypertrophic chondrocytes (Zhao *et al.* 1997). This expression parallels that of the gene for type II collagen (Col2a1), a specific marker of chondrocyte differentiation.

1.5. Skeletal muscle resident mesenchymal stromal cell populations

It was originally suggested that satellite cells had the ability to differentiate into lineages other than the myogenic lineage, but recent studies have demonstrated that they are committed to the myogenic lineage and do not spontaneously adopt non-myogenic fates (Starkey *et al.* 2011). Also, several reports suggest that stem/progenitor cell populations other than satellite cells participate in skeletal muscle repair/regeneration. Several groups have identified muscle-resident mesenchymal progenitor cells with varying levels of multipotency from mouse and human skeletal muscle. Interestingly, all these described populations share a common characteristic: to be closely associated with small blood vessels within the interstitial tissue (Crisan *et al.* 2008; Crisan *et al.* 2012). Importantly, one must keep in mind that most of the different published sub-populations may be directly related to each other and/or share a common progenitor although they were not isolated and cultured the same way. Below are the main muscle resident mesenchymal progenitors described to date.

1.5.1. Pericytes

Pericytes are defined by their location, closely encircling endothelial cells in small vessels and capillaries (Andreeva *et al.* 1998). Recent studies suggest that pericytes include progenitors of different cell types, capable of differentiating along several lineages including osteogenic and chondrogenic (Collett and Canfield 2005). However these populations represented pericyte-containing cultures and not purified pericytes. Dellavalle *et al.* demonstrated that pericytes sorted from human skeletal muscle based on alkaline phosphatase (AlkP) expression are myogenic precursors, distinct from satellite cells, which support skeletal muscle regeneration (Dellavalle *et al.* 2007). Also, $AlkP^+$ pericyte-derived cells can differentiate into osteoblasts and adipocytes *in vitro*.

Pericytes were sorted from multiple human organs, including fetal and adult skeletal muscle, by FACS. These perivascular cells were identified and sorted based on $CD146^+$ expression, whereas contaminating myogenic ($CD56^+$), hematopoietic ($CD45^+$) and endothelial ($CD34^+$) cells were eliminated. Sorted human perivascular cells ($CD146^+CD56^-CD45^-CD34^-$) expressed recognized markers of mesenchymal stem cells, including CD73, CD90 and CD105. Additionally, these cells can be expanded *in vitro*. Also, when cultured in appropriate inductive conditions, the sorted perivascular cells differentiated into chondrocytes, adipocytes and osteocytes at the clonal level *in vitro* and developed into bony nodules when transplanted into a skeletal muscle pocket in a murine model (Crisan *et al.* 2008).

1.5.2. Muscle-derived stem cells (MDSCs)

Murine skeletal muscle contains a population of stem cells termed muscle-derived stem cells (MDSCs) (Qu-Petersen *et al.* 2002). MDSCs are isolated from the muscle homogenate using a pre-plating technique, which eliminates the contaminating more adherent cell types (Gharaibeh *et al.* 2008). The more slowly adherent MDSCs have demonstrated enhanced differentiation potential, and can be induced to become osteoblast, adipocytes and chondrocytes *in vitro*. Also, these MDSCs have the ability to differentiate into myoblasts *in vitro* and to promote muscle regeneration *in vivo*. Interestingly, a

comparable population of MDSCs has not been harvested from human muscle tissue solely on the basis of their adhesion characteristics.

1.5.3. Myoendothelial cells (MECs)

In addition to satellite cells ($CD56^+$) and endothelial cells ($CD34^+CD144^+$), human skeletal muscle contains a rare (<0.5%) population of cells that coexpress myogenic and endothelial cell markers ($CD56^+CD34^+CD144^+$), which has been identified using FACS to isolate the cells that are positive for $CD56^+CD34^+CD144^+$. Myoendothelial cells (MECs) proliferate long term, retain a normal karyotype, and survive better under oxidative stress than myogenic ($CD56^+$) cells. Although most satellite cells are committed to the myogenic lineage, the small subset of satellite cells that co-expresses the endothelial markers is capable of multilineage differentiation into myogenic, chondrogenic, and osteogenic cells *in vitro*. Also, these cells appear to have enhanced myogenic potential *in vivo*, participating in myofiber regeneration. MECs are associated with the vasculature in human skeletal muscle, specifically located in the intimal compartment of blood vessels within human skeletal muscle (Zheng *et al.* 2007).

A major limitation in characterizing the multipotent potential of MECs was the likely heterogeneous nature in "stemness", therefor it was investigated whether chondrogenic and osteogenic potential was preserved at the clonal level. Clonal myoendothelial cells (cMECs: $CD56^+CD34^+CD144^+CD45^-$) were purified (from fresh human muscle biopsies by FACS. cMECs displayed robust multipotency *in vitro* and *in vivo* (Zheng *et al.* 2013), including chondrogenesis, osteogenesis, and adipogenesis, in addition to myogenesis. cMECs seem to represent a human counterpart of murine MDSCs, as one of the multilineage mesodermal stem cell populations residing in the vascular niche within the adult skeletal muscle.

1.5.4. Skeletal muscle mesenchymal progenitors

The PDGFRα was used to identify mesenchymal progenitors in adult skeletal muscle, responsible for ectopic fat cell formation (Uezumi *et al.* 2010). PDGFRα$^+$ mesenchymal progenitors are distinct from satellite cells and are located in the muscle interstitium. The expression of PDGFRα appears to be a characteristic of undifferentiated MSCs (Ball *et al.* 2007). According to Uezumi *et al.*, of the muscle-derived cell populations, only PDGFRα$^+$ cells show efficient adipogenic differentiation both *in vitro* and *in vivo* (Uezumi *et al.* 2010). In a subsequent study, it was shown that PDGFRα$^+$ mesenchymal progenitors could also differentiate into ECM-producing cells. Clonal analysis showed that these cells possess the ability to differentiate into both collagen type-I-producing cells and adipocytes (Uezumi *et al.* 2011).

Moreover, murine fibro/adipogenic progenitors (FAPs) sorted by FACS (Lin$^-$α7$^-$Sca-1$^+$) efficiently generate both fibroblasts and adipocytes at the clonal level, but fail to generate other mesenchymal lineages. Interestingly, Lin$^-$α7$^-$Sca-1$^+$ cells were uniformly PDGFRα$^+$ (Joe *et al.* 2010).

Recent lineage tracing studies have implicated the vascular endothelium in HO, as cells expressing the angiopoietin receptor, Tie2, robustly contribute to all stages of BMP-induced skeletogenic lesions (Lounev *et al.* 2009; Medici *et al.* 2010). A population of Tie2$^+$PDGFRα$^+$Sca1$^+$ cells was identified as multipotent mesenchymal progenitors that reside in the murine skeletal muscle interstitium and represent a significant cell of origin for HO. These progenitors reside in the interstitium of skeletal muscle and other tissues, and are distinct from the endothelium. Intramuscular transplantation, together with clonal analysis in culture, revealed that these progenitors are multipotent, exhibiting the capacity for both BMP-dependent skeletogenic differentiation and spontaneous adipogenic differentiation (Wosczyna *et al.* 2012).

Recently, a group suggested that PDGFRα$^+$ mesenchymal progenitors residing in human skeletal muscle are the major cell origin of HO. In this study, both CD56$^+$ myogenic cells and PDGFRα$^+$ mesenchymal progenitors showed comparable osteogenic

differentiation potential *in vitro*. However, in an *in vivo* ectopic bone formation model, only PDGFRα^{+} cells formed bone-like tissue and showed successful engraftment (Oishi *et al.* 2013)

1.5.5. Muscle-resident stromal cells (mrSCs)

Our laboratory identified and isolated a population of murine muscle-resident stromal cells (mrSCs) which are also closely associated with the vasculature (Scime 2005, Leblanc 2011). The common stromal cell marker Sca1 was used to identify the mrSC population, whereas hematopoietic (Lin^{+}) and endothelial (CD31^{-}) cell lineages were discarded. Depending on their culture conditions, this cell population took on an endothelial (Grenier *et al.* 2007), an adipogenic (Scime *et al.* 2005), a fibrogenic (Trensz *et al.* 2010) or an osteogenic fate (Leblanc *et al.* 2011). This subpopulation is also augmented in chronic (Trensz *et al.* 2010) and acute muscle damage (Grenier *et al.* 2007; Leblanc *et al.* 2011), a condition which is known to lead to fibrosis and fatty degeneration. Not only are they known to express RunX2 and AlkP during osteogenic differentiation *in vitro*, mrSCs have been shown to contribute to HO *in vivo* in a murine model for HO which consisted of injecting BMP9 within a damaged muscle (Leblanc *et al.* 2011).

1.5.6. Traumatized muscle mesenchymal progenitor cells

A population of multilineage mesenchymal progenitor cells was identified in human traumatized muscle, capable of undergoing osteogenic differentiation *in vitro* (Nesti *et al.* 2008). These cells were isolated and enriched from digested muscle tissue based on their high adhesion characteristics to tissue culture plastic. Moreover, they have a cell surface epitope profile that is characteristic of BM-MSCs (Jackson *et al.* 2009), although they appeared to demonstrate limited lineage commitment compared to their bone marrow counterparts. It was shown that damage triggers the activation and proliferation of a higher number of progenitors within muscle and that those cells were more responsive to osteogenic cues (Jackson *et al.* 2011)

The ongoing research is focused on improving skeletal muscle regeneration, and limiting regenerative disorders, by investigating the hypothesis that cells related to the vascular wall in muscle tissue can (under appropriate conditions) give rise to potent "stem" cells that possess strong multilineage potential. For the time being, there is a lack of consensus in the field on the appropriate MSC isolation technique, caused largely by the absence of a truly selective and universally adopted MSC marker, especially in human skeletal muscle.

Table 1: Summary of the different mesenchymal progenitor cell populations described from skeletal muscle

Name	Species	Enrichment	Localization	Lineage potential	Reference
[illegible]					
Fibro/adipogenic progenitors (FAPs)	Mouse	**FACS** Lin^- $\alpha7^-$ $Sca1^+$	Muscle interstitium Closely associated with blood vessels	Clonal multipotence (*in vitro* and *in vivo*) - adipogenic - fibrosis	(Joe *et al.* 2010)
[illegible]	[illegible]	[illegible]			[illegible]
Muscle-resident stromal cells (mrSCs)	Mouse	**FACS** $Sca1^+$ Lin^- $CD31^-$		*In vitro* : - endothelial, - adipogenic, - chondrogenic - osteogenic *In vivo*: - osteogenic	(Grenier *et al.* 2007; Trensz *et al.* 2010; Leblanc *et al.* 2011)
[illegible]					
Muscle-derived stem cells (MDSCs)	Mouse	**Pre-plate technique** (slow adhering)	Myofiber periphery closely associated to blood vessels	*In vitro* multipotence - myogenic - osteogenic - chondrogenic - adipogenic *In vivo* muscle regeneration	(Qu-Petersen *et al.* 2002)

Myoendothelial cells (MECs)					
Myoendothelial cells (MECs)	Human	**FACS** $CD56^+$ $CD34^+$ $CD144^-$	Interstitial space between myofibers, associated with the vasculature	*In vivo* muscle regeneration *In vitro* multipotence: - myogenic - chondrogenic - osteogenic	(Zheng *et al.* 2007)
[illegible]	[illegible]	[illegible]		[illegible]	[illegible]
Perivascular cells					
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Pericytes	Human	**FACS** $CD146^+$ $CD34^-$ $CD45^-$ CD56-	Periphery of capillaries and microvessels	*In vitro* clonal multipotence - osteogenic - chondrogenic - adipogenic - myogenic *In vivo* - osteogenic - myogenic	(Crisan *et al.* 2008)

1.6. Other skeletal muscle progenitor populations

1.6.1. Brown adipocytes

Humans have two types of adipose tissue: white adipose tissue (WAT), which not only stores energy in the form of triglycerides but is also recognized as an important endocrine and immune organ, and brown adipose tissue (BAT), which is specialized in energy expenditure by burning lipids to generate heat (thermogenesis). While WAT structure is characterized by a single, large, lipid droplet *in vivo* and few mitochondria, BAT contains several small lipid droplets (multilocular), many mitochondria, and uniquely expresses high levels of uncoupling protein 1 (UCP1) (Cannon and Nedergaard 2010; Nedergaard *et al.* 2010; Richard and Picard 2011). UCP1 is a proton channel of the inner mitochondrial membrane that allows proton influx into the mitochondrial lumen. UCP1 acts to uncouple oxidative phosphorylation from ATP production, thereby releasing energy as heat (termed thermogenesis). BAT plays a pivotal role in adaptive thermogenesis, a physiological process during which energy is dissipated in response to environmental changes, such as cold temperature and diet (Lowell and Spiegelman 2000).

1.6.1.1. Origin of BAT

It has been suggested that BAT and skeletal muscle may share a common developmental ancestry (Timmons *et al.* 2007) . Myf5-expressing progenitors can give rise to both skeletal muscle and interscapular brown fat (Seale *et al.* 2008). In accordance with these observations, BAT progenitors have also been identified in human skeletal muscle (Crisan *et al.* 2008). These cells provide the potential for increasing the oxidative capacity of these tissues by targeting these endogenous precursor cells to differentiate *in vivo* into energy-dissipating brown adipocytes. Though, it is now clear that two different types of brown adipocytes exist, which have distinct developmental origins. Classical brown adipocytes residing in the interscapular and perirenal regions develop from myofibroblastic-like Myf5-positive precursors (Seale *et al.* 2008) that differentiate into brown adipocytes through the action of transcriptional regulators PRDM16 and C/EBPb (Kajimura *et al.* 2009); whereas, pockets of a second, distinct type of UCP1-positive

adipocyte are found sporadically in the WAT of adult animals that have been exposed to chronic cold or β-adrenergic stimulation. These inducible brown-like adipocytes (beige or brite cells) possess many of the biochemical and morphological characteristics of classical brown adipocytes, including the presence of multilocular lipid droplets (Frontini and Cinti 2010). However, they arise from a non-Myf5 cell lineage, hence, have a distinct origin. Also, it has been shown that epididymal WAT-derived brite cells that are induced by rosiglitazone (ROS) do not express myocyte-enriched genes (Petrovic *et al.* 2010).

1.6.1.2. Factors regulating human brown adipogenesis

Among factors regulating human brown adipogenesis, most of them have been identified and characterized in mouse models *in vitro* as well as *in vivo*. Within these signaling pathways, Hedgehog and BMP7 were described as modulators of brown adipogenesis (Tseng *et al.* 2008; Pospisilik *et al.* 2010; Schulz *et al.* 2011). When tested on human multipotent adipose derived stem cells (hMADs) as a model for brown adipogenesis, both Hedgehog and BMP7 did not have any significant effect on hMADs "brite" conversion, indicating discrepancies between mouse and human brown adipocyte regulation (Pisani *et al.* 2011). However, it has been shown that the activation of PPARγ by synthetic ligands induces a brown fat-like gene program in WAT (Fukui *et al.* 2000; Sell *et al.* 2004; Rong *et al.* 2007; Petrovic *et al.* 2010). Among members of the thiazolidinedione family, ROS is known as a high-affinity ligand of PPARγ. Mechanistically, these drugs function by directly binding to and activating PPARγ and PPAR-response elements (PREs) on the promoter and/or enhancer of brown fat-selective genes (Sears *et al.* 1996; Viswakarma *et al.* 2007).

Recent research has also identified several dominant transcriptional regulators of brown adipocyte development and function, including peroxisome proliferator activated receptor gamma coactivator 1α (PGC1alpha) and PRD1-BF-1-RIZ1 homologous domain containing protein-16 (PRDM16) (Kajimura *et al.* 2010). PGC-1α is highly expressed in BAT compared with WAT and is responsible for regulating mitochondrial biogenesis and thermogenesis. Mice deficient in PGC-1α are cold-sensitive with low expression of UCP1 and a morphologically abnormal BAT (Lin *et al.* 2004). It is generally accepted that PGC-

1α is the critical regulator of adaptive thermogenesis in responsive tissues. PRDM16 on the other hand is a 140-kDa zinc-finger PR domain-containing protein that induces a program of gene expression as well as mitochondrial biogenesis/oxygen consumption, consistent with the brown phenotype, when ectopically expressed in white preadipocytes in culture or white depots in the animal (Seale *et al.* 2007). Also, PPARγ functions to regulate the underlying adipogenic programs common to both white and brown adipocytes. It is possible that its activity can favor one phenotype over the other. Several recent studies have shown that exposure of white adipocytes, either in culture or in animals, to potent PPARγ ligands such as ROS induces a "browning" of the white cells, as characterized by an increase in mitochondrial mass and structure as well as a markedly enhanced oxygen consumption and lipid oxidation (Wilson-Fritch *et al.* 2003; Wilson-Fritch *et al.* 2004). This process is likely due to PPARγ ligand-associated induction of mitochondrial genes, including UCP1 and cytochrome c oxidase (Cox) (Wilson-Fritch *et al.* 2004).

1.6.1.3. Adult BAT

In humans, BAT is present in abundant quantity in newborns (Lean *et al.* 1986), but it was traditionally believed that BAT was nonexistent or non-functional in adult humans. However, this dogma was reversed by evidence from nuclear medicine (Nedergaard *et al.* 2007; Cypess *et al.* 2009; Saito *et al.* 2009; van Marken Lichtenbelt *et al.* 2009; Virtanen *et al.* 2009), which showed active BAT in adult humans. Indeed, fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET) studies allowed visualization in humans of highly dynamic adipose tissue depots. Their metabolism was stimulated by cold. These depots were proposed to represent BAT that had been undetected until now (Nedergaard *et al.* 2007). Since then, there has been a flurry of new data surrounding BAT function and therapeutic potential (Enerback 2010; Ravussin and Galgani 2011; Muller and Bosy-Westphal 2013).

1.6.1.4. Brown adipocyte progenitor populations in skeletal muscle

In adult humans, it remains to be determined which population of progenitors gives rise to brown fat. Although a group identified a subpopulation of adipogenic progenitors

(Sca-1$^+$/CD45$^-$/Mac1$^-$; referred to as Sca-1$^+$ cells) residing in murine brown fat, white fat, and skeletal muscle. These Sca-1$^+$ cells from skeletal muscle and subcutaneous white fat are highly inducible to differentiate into brown-like adipocytes upon stimulation with BMP7 (Schulz *et al.* 2011).

1.6.2. Myofibroblasts

Following tissue injury, fibroblasts are recruited during the inflammatory phase of tissue regeneration. These cells are then activated and acquire a smooth muscle cell-like phenotype; they are consequently called myofibroblasts. These myofibroblastic cells synthesize and deposit ECM components. They also exhibit contractile properties, due to the expression of α-smooth muscle actin (αSMA) in microfilament bundles or stress fibers, playing a major role in the contraction and maturation of the granulation tissue (Hinz 2010). The presence of αSMA represents the most reliable marker of the myofibroblastic phenotype (Tomasek *et al.* 2002). During the final phase of healing, proteolytic enzymes, essentially matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinases [TIMPs]) play an essential role. Following which the number of vascular cells and myofibroblasts is dramatically reduced by the process of apoptosis (Desmouliere *et al.* 1995). Persistent fibrosis is often due to an imbalance between ECM synthesis and degradation by myofibroblasts due to chronic inflammation.

1.6.2.1. TGFβ1 and myofibroblastic differentiation

Several cytokines and growth factors have a role in wound healing. Among these, TGFβ1 is a potent inducer of myofibroblastic differentiation (Desmouliere *et al.* 1993). Beyond induction of αSMA expression, TGFβ1 also promotes the deposition of large amounts of ECM and reduces MMP activity by promoting TIMP expression (Micallef *et al.* 2012). Hence, TGFβ1 plays a key role in inducing the formation of fibrotic tissue that limits muscle healing after severe injury.

1.6.2.2. Myofibroblast identification

One limitation that has hindered studies of fibrosis is the lack of good markers to label fibroblasts/myofibroblasts. It is well established that in non-muscle systems, activated fibroblasts may be identified by their increased proliferation, migratory ability, enhanced contractility, increased expression of vimentin and, in particular, αSMA, a contractile protein of stress fibers. When αSMA stress fibers contract, they exert mechanical tension on the ECM, which in turn provides a mechanically resistant support, hence the name "myofibroblast". These cells are associated with tissue repair and fibrosis in many tissues and organs, including muscle, skin, liver, lung, bone and cartilage (Hinz *et al.* 2007). However, despite their relevance in these diseases, it remains unclear whether myofibroblasts are present in fibrotic skeletal muscle, or whether they are instead mature fibroblasts actively producing ECM components (Lieber and Ward 2013). The identification of the myofibroblastic progenitor in skeletal muscle would help determine their role in fibrosis.

2. HYPOTHESIS

The identity and regulation of the multipotent progenitors residing within the skeletal muscle are topics of great interest and intense study. Developing a method to obtain an enriched muscle resident stromal cell population from human skeletal muscle would help understand their role during normal skeletal muscle regeneration as well as the mechanisms behind their aberrant activation during regenerative disorders. Furthermore, the multipotent capabilities of these cells also make them excellent candidates for potential applications in cellular therapy and/or tissue engineering.

We believe that FACS can be used as an efficient enrichment method, to isolate the stromal subpopulation from the adherent fraction of human muscle-derived cells by using multiple mesenchymal stromal cell markers identified in other tissue sources. We propose that these markers will enable the identification of the human mrMSC subset as they do in other tissue sources and enable the isolation of a cell subpopulation with superior multipotent capabilities. Furthermore, we propose that a defined culture system that enables the proliferation and maintenance of these cells in culture will be more compatible with future applications and foster inter-laboratory reproducibility.

To test this hypothesis, we have established three objectives:

3. OBJECTIVES

1) Develop a reproducible enrichment strategy for the isolation and the culture of the adherent human muscle resident mesenchymal stromal cell fraction

2) Identify the multipotent stromal cell subpopulation

3) Characterize other progenitor populations present in human skeletal muscle

4. MATERIAL AND METHODS

4.1. Human adult skeletal muscle samples

Healthy human muscle tissue (gracilis and semitendinosus) **(Fig. 4.1)** was obtained from patients (mean age, 32 years; range, 18 to 48 years; 56% male and 44% female) undergoing anterior cruciate ligament (ACL) reconstruction surgery at the Centre Hospitalier Universitaire de Sherbrooke (CHUS) **(Table 2)**. The protocol was reviewed and approved by the CHUS Ethics Committee (#11-122), and the biopsy was performed under the informed consent. Muscle samples (mean mass, 5.4±1.2g) were placed on ice in sterile tissue culture medium composed of Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics, and immediately transferred to the laboratory.

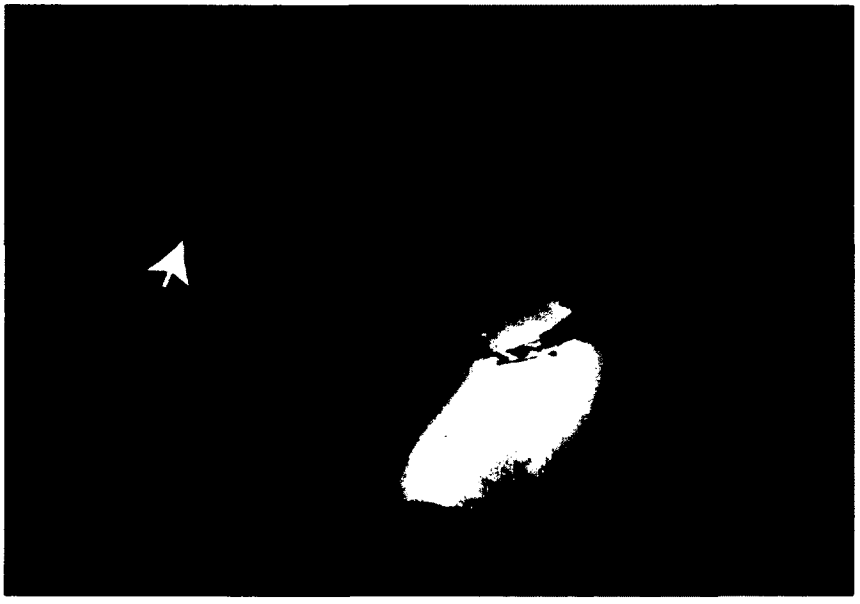


Figure 4.1: Semitendinosus tendon with associated muscle tissue (arrowhead).

Table 2: Summary of human muscle biopsies used for experiments

Sample No	Sex	Age (years)	Weight (g)
M37	M	21	3.84
M38	F	37	3.23
M39	F	28	4.49
M41	M	24	6.30
M52	F	41	7.35
M58	M	36	7.35
M60	F	52	5.88
M63	M	21	5.72
M68	M	34	4.58
Average		**32.7±8.1**	**5.4±1.2**

4.2. Tissue preparation

Skeletal muscle samples were carefully dissected leaving them free of any tendon and/or fat tissues. They were then minced and digested with 1 mg/mL collagenase type I (Sigma) in DMEM containing 10% FBS, for 30 min at 37°C at a ratio of 4g of tissue for 30ml of 1mg/ml collagenase type I in medium. The tissue slurry was diluted with medium and passed through a 70-μm and then a 40-μm cell strainer (Becton Dickenson) prior to centrifugation (325xg, 6 min, 4°C). Cell pellets were resuspended in 8ml of fresh medium.

4.3. Cell culture

Primary human muscle derived adherent cells were grown in Mesencult-XF® medium (StemCell Technologies, Vancouver, Canada) supplemented with 1% antibiotics. This medium is referred to as 'defined medium'. Collected cells were counted and allowed to attach in a 100-mm tissue culture plate coated with Mesencult-XF® attachment substrate (StemCell Technologies) for approximately 7 days without a medium change on day 4. The cells isolated from approximately 4g of tissue were left to adhere in a single 100mm petri dish. Roughly $7x10^5$ adherent cells were recovered per gram of tissue. At 80% confluence,

cells were trypsinized, centrifuged (325xg, 6 min, 4°C) and resuspended in defined medium as passage 1 cells with fresh medium changes every 3-4 days. Cells were sub-cultured at a density of $4x10^3$ cells/cm^2.

Normal human dermal fibroblasts (NHDF) (ATCC, Manassas, VA, USA) were grown in DMEM supplemented with 10% FBS and 1% antibiotics in uncoated 100-mm tissue culture plates.

Human skeletal muscle myoblasts (HSMM) (Lonza, Allendale, NJ, USA) were cultured in Clonetics™ skeletal muscle cell growth media (Lonza, Allendale, NJ, USA) according to the manufacturer's recommendation. Cells were passaged at 80% confluence.

4.4. Fluorescent-activated cell sorting of human muscle resident cells

First passage cells were detached with the Accutase™ Cell Detachment solution (BD Biosciences), centrifuged and resuspended in cold sorting buffer (PBS; 1mM EDTA; 25 mM HEPES pH 7.0; 1% FBS) at ~$1x10^6$ cells per ml. The cells were incubated for 20 min. on ice with appropriate primary antibodies **(Table 3)** according to the manufacturers' instructions. During the cell sorting experiment live cells were distinguished from dead cells with the LIVE/DEAD® Violet Viability/Vitality Kit (Invitrogen). Fluorescence compensation was done using the BD CompBeads Set Anti-Mouse Ig, κ (BD Biosciences). Sorting was performed using a BD FACSAria™ cell sorter (BD Biosciences) equipped with four lasers, using a 100-μm nozzle at 20 psi. Sorting gates were defined based on unstained controls. Analysis was performed using FlowJo 7.9 software (Treestar Inc., Ashland, OR, USA). A population of unsorted cells was also kept as a control. Unsorted and sorted fractions were then expanded in defined medium on coated tissue culture dishes.

Table 3: Summary of primary antibodies used for flow cytometry

Antibody	Clone	Isotype	Company
FITC anti-human CD90	5E10	Mouse IgG1, κ	BD; 555 595
APC anti-human CD73	AD2	Mouse IgG1, κ	BD; 560 847
PerCP-CyTM 5.5 anti-human CD105	266	Mouse IgG1, κ	BD; 560 819
PE-Cy7 anti-human CD31	WM59	Mouse IgG1	eBioscience; 25-0319
PE anti-human CD34	581	Mouse IgG1, κ	BD; 562 799
BV421 anti-human CD140a	αR1	Mouse IgG2a, κ	BD; 562 799

N.B.: The dilution used for each antibody was made according to the manufacturer's instructions.

4.5. *In vitro* differentiation (osteogenic, adipogenic and chondrogenic)

For adipogenic and osteogenic assays, cells (passage 5 or 6) were seeded at a density of 8 x 10^3 cells per well in 24-well collagen-coated plates in defined medium until the cells reached confluence, typically 4-5 days after seeding. The tissue culture grade plates were coated with 0.1 mg/ml rat-tail collagen type 1 (Millipore) for 2 hours and dried overnight before plating.

For osteogenic differentiation cells were stimulated with osteogenic medium (see medium composition summarized in **Table 3**) for 21 days as described before with some modifications (Lecourt *et al.* 2010; Lee *et al.* 2010). Cells of the control group were cultured in DMEM supplemented with 5% horse serum (HS). To assess mineralization, calcium deposits in cultures were stained with Alizarin Red-S (Sigma; 40mM pH 4.1) (Gregory *et al.* 2004).

For adipogenic differentiation, cells were first treated with an adipogenic induction medium for 72h, followed by 18 days of white adipogenic growth medium, a protocol adapted from Vermette *et al.* (Vermette *et al.* 2007). Differentiation media was changed twice a week for 3 weeks. An Oil Red O solution (Sigma; 0.5% Oil red O in isopropyl

alcohol) was used to detect triglycerides present within lipid droplets of mature adipocytes **(Table 4)**.

The same protocol was used for brown adipocyte differentiation, with the exception of continuous ROS (Sigma) stimulation, adapted from Pisani *et al.* (Pisani *et al.* 2011), and only 11 days of brown adipocyte growth medium. BMP7 was also added to the adipogenic differentiation medium in order to determine its capacity to induce brown versus white adipogenesis (Tseng *et al.* 2008; Schulz *et al.* 2011).

For chondrogenesis, chondrogenic culture medium consisting of serum-free DMEM containing 10μM dexamethasone (Sigma), 1% ITS+ (Wisent) and 10ng/ml TGFβ1 (Sigma) was added to ~$2.5x10^5$ cells (passage 5 or 6) in a sterile 15-ml tube which were pelleted by centrifugation at 350xg for 6 min at 4°C. It is important to add the chondrogenic medium before centrifugation, since the pellet formed after centrifugation could be resuspended by adding medium. Each pellet was cultured in 0.5μl of media which was changed twice a week. The pellets were harvested on day 21, fixed in 4% phosphate buffered formalin, processed into paraffin wax, sectioned at 5μm and stained with Alcian blue (ACROS ORGANICS; 1% Alcian blue in 3% acetic acid) which stains the highly sulfated proteoglycans that characterize the cartilaginous matrix **(Table 4)**.

Table 4: Summary of the multilineage differentiation media composition

Lineage	Basal medium	Induction supplements	Assay
Osteogenic	DMEM, 5% HS 1% antibiotics	10 µg/ml ascorbic acid 100 nM dexamethasone 10 mM β-glycerophosphate	Alizarin red S
Chondrogenic	DMEM, 1% antibiotics	10 µM dexamethasone 1X ITS 10 ng/ml TGFβ1	Alcian blue
Adipogenic Induction	DMEM, 3% FBS 1% antibiotics	100 nM Insulin 0.2 nM T_3 1 µM dexamethasone 0.25 mM isobutyl methylxanthine 1 µM ROS	Oil Red O
White adipogenic growth	DMEM, 10% FBS, 1% antibiotics	100 nM Insulin 0.2 nM T3 1 µM dexamethasone	
Brown adipogenic growth	DMEM, 10% FBS, 1% antibiotics	100 nM Insulin 0.2 nM T3 1 µM dexamethasone 1 µM ROS	
Myofibroblastic	DMEM, 2% HS, 1% antibiotics	25 ng/ml TGFβ1	αSMA

4.6. Clonal growth and differentiation

Clones of the multipotent human muscle resident mesenchymal stromal cell (hmrMSCs: [$CD73^+CD105^+CD90^-$]) were established by limiting dilution. Briefly, passage 2 cells were suspended at a concentration of less than 1 cell per 200 µl of defined medium

and plated in coated 96-well plates with 200 µl of medium per well and incubated at 37°C and 5% CO_2, with medium changes once a week. After 72h, wells with a single cell were identified. After 2-3 weeks, single cell-derived clones were treated with 0.25% trypsin and seeded into 48-well plates, and subsequently 24-well plates to expand into clonal progenies. Clonal progenies were transferred to 6-well plates upon confluence of approximately 80% and maintained with medium changes every 3-4 days.

4.7. RNA extraction

Total RNA was extracted using TRIzol® (Invitrogen) according to the manufacturer's instructions with a few modifications. The cells were rinsed with cold (4°C) PBS and then 500 µl of TRIzol® was added to each sample. The cell lysate was recovered with a sterile cell scrapper and transferred to a microtube. Then, 100 µl of chloroform was added and the samples were vigorously agitated by hand for 15 sec. and centrifuged (13.2K, 15 min., 4°C). The RNA was precipitated 1h at 4°C with isopropanol and 1 µg of glycogen, rinsed with ethanol and resuspended in RNAse-free water. The samples were kept at -80°C for up to one month.

The concentration of RNA was determined by measuring the absorbance at 260nm, were 1 unit of absorbance (Abs) is equal to a concentration of 0.025 mg/ml (Commoner and Lipkin 1949; Kocsis *et al.* 2006). The A260/A280 ratio was calculated to ensure purity of the RNA. A ratio superior to 1.7 was considered acceptable.

4.8. Complementary DNA and reverse transcription

The RNA (1 µg) was reverse-transcribed using the RT Superscript II kit (Invitrogen). Following the reaction, 60 µl of sterile water was added to each tube for a final concentration of 25 ng/µl of cDNA.

4.9. Quantitative PCR (qPCR)

The reactions were prepared in a total volume of 20 µl with SYBR green master mix 2X (BioRad), 50 ng of cDNA (2µl) and 50 ng of each primer. The samples were then

placed in the qPCR device (RotorGene 6000, Corbett Robotics, Eight Mile Plains, Australia) and the reactions executed with the following program: 10 min at 95°C; 40 cycles of 40 seconds at 95°C and 40 seconds at 56°C. The dissociation curve (melting curve) was verified each time to ensure primer quality, such as specificity as well as the absence of primer-dimers. The results were analyzed using the $2^{-\Delta\Delta CT}$ relative quantification method normalized to the TATA-box binding protein (TBP). The primer sets are listed in **Table 5** and were obtained from a previous study (Pisani *et al.* 2011).

Table 5: Primer sequences used for gene expression analysis

Gene	Forward primer	Reverse primer	Accession No
PPARγ	AGCCTCATGAAGAGCCTTCCA	TCCGGAAGAAACCCTTGCA	NM005037
FABP4	TGTGCAGAAATGGGATGGAAA	CAACGTCCCTTGGCTTATGCT	NM_001442.2
ADIPOQ	GCAGTCTGTGGTTCTGATTCCATAC	GCCCTTGAGTCGTGGTTTCC	NM 004797.3
UCP1	GTGTGCCCAACTGTGCAATG	CCAGGATCCAAGTCGCAAGA	NM021833
CIDEA	GGCAGGTTCACGTGTGGATA	GAAACACAGTGTTTGGCTCAAGA	NM001279
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC	NM003194

4.10. Immunofluorescence

Cells were fixed in 4% paraformaldehyde (PFA) diluted in PBS (10 min at 4°C), blocked in PBS containing 10% goat serum, 1% BSA, and 0.2% Triton®X-100, and then incubated with anti-Pax7 (1:3, DSHB), anti-UCP1 antibody (1:800, H-150, Abcam) or anti-human α-smooth muscle actin (αSMA) antibody (1:100, 1A4, Cedarlane) for 90 min. at room temperature (RT). After several rinses in PBS-Tween-20 (0.1%), cells were incubated with either Alexa Fluor®488-conjugated goat anti-mouse IgG or Alexa Fluor®594-conjugated goat anti-rabbit IgG secondary antibody (1:1000, Invitrogen). Primary antibodies were omitted as a control. Cell nuclei were stained with DAPI reagent (Sigma-Aldrich) for 10 min after secondary antibody incubation. Indirect immunofluorescence was examined without counterstaining using an Axioskop 2 phase-contrast/epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY, USA) or using a DMIRE2 inverted microscope (Leica Microsystems). Photomicrographs were captured using a Retiga SRV

cooled color digital camera (Qimaging, Burnaby, Canada). The images were processed using Adobe Photoshop CS5 (Adobe suite).

4.11. Western blot analyses

Cells were lysed on ice in RIPA buffer (0.5% (v/v) NP-40, 0.1% (m/v) SDS, 150 mM NaCl, 50m M Tris-HCl, pH 7.5) containing protease inhibitors (Complete™, Roche Molecular Biochemical). The homogenate was centrifuged 15 min at 4°C and the supernatant containing the proteins recovered. The protein concentration was determined by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as a standard protein.

The protein extracts (30 µg) were prepared in Laemmli loading buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% (m/v) SDS, 0.01% (m/v) bromophenol blue, 10% (m/v) β-Mercapto-ethanol) and were separated by polyacrylamide gel electrophoresis (PAGE). Protein separation was done at a constant voltage of 100V for 90 min. Then, the proteins were transferred to PVDF membranes (Millipore), previously activated in methanol, in transfer buffer (150 mM glycine, 20 mM Tris-base and 10% (v/v) methanol). The transfer was done at a constant voltage of 80V for two hours. The membranes were then rinsed three times in PBS-T (PBS with 0.1% (v/v) Tween20) and incubated with anti-UCP1 (1:1000, 145-159, Abcam) and anti-GAPDH (1:1000, FL-335, Santa-Cruz) antibodies overnight at 4°C. The membranes were again rinsed in PBS-T before being incubated with the secondary HRP (horseradish peroxidase) coupled antibody (1:5000; Amersham) at RT for one hour. After several rinses the membranes were exposed by chemiluminescence, with a homemade ECL solution (unpublished). This solution is a 1:1 mix of solution A and B. The solution A contains 10 ml of Tris 0.1M pH 8.5, 50 µl of 90 mM coumaric acid in DMSO and 100 µl of 250 mM luminol in DMSO. The solution B is made up of 10 ml of Tris 0.1 M pH 8.5 and 6 µl of 30% (v/v) hydrogen peroxide. Solutions A and B are combined and deposited on the membrane for 1 min. Biomax ML film (Kodak) is placed against the membranes in order to create an autoradiogram of the antibody binding sites, which is digitized. The bands were quantified by densitometry with the ImageJ software (version 1.46, National Institute of Health, USA) (Abramoff et al. 2004).

4.12. Myofibroblastic differentiation

For myofibroblastic differentiation, cells (passages 3 to 6) were seeded at a density of $4x10^3$ cells per cm^2 on collagen-coated plates (as described above) in defined medium until the cells reached 75% confluence, typically 2 days after seeding. Myofibroblastic differentiation medium consisted of DMEM, supplemented with 2% HS and 5 ng/ml or 25 ng/ml of recombinant human TGFβ1 (Sigma) as described elsewhere (Lee *et al.* 2010). For the negative control condition, the TGFβ1 was omitted **(Table 4)**.

4.13. Collagen gel contraction assay

Cells grown in defined medium were trypsinized, centrifuged (325xg, 6 min, 4°C) and resuspended in DMEM supplemented with 0.5% FBS and kept on ice. 250 000 cells were resuspended in 500 µl of a 2 mg/ml collagen solution (rat tail collagen, Type 1, BD) according to manufacturer's instructions. The cell-populated collagen solution was placed in 48-well plates (500 µl per well) and placed at 37°C for 30 min to induce gelation. 500µl of the appropriate induction medium was then added to each well (described above) and the cells were incubated in a humidified atmosphere at 37°C. After 48h of incubation, cell populated collagen lattices were physically detached and allowed to float freely in the medium. The diameters of the collagen lattices were digitalized at several times points for up to 4 days and were measured using the ImageJ software (Abramoff et al. 2004).

4.14. Statistical Analysis

The values were expressed as mean +/- standard error of the mean (SEM). Statistical analyses were performed using the paired *t*-tests for experiments comparing same cell lines from one experiment. Unpaired *t*-tests were used for the experiments comparing different cell lines or the same cells from different experiments. Power calculations were performed with a confidence interval of 95%. The $p<0.05$ values were considered statistically significant. Statistical analyses and graphics were carried out using the Prism 5.0 software (GraphPad Software Inc.).

5. RESULTS

5.1. Enrichment strategy for human muscle resident mesenchymal stromal cells

5.1.1. Isolation and culture conditions

Freshly isolated human muscle resident cells were plated and cultured as adherent cells. In order to determine the optimal growth medium, we cultured cells with a classic growth medium containing 10% FBS and another commercially available defined medium which did not contain serum. Cells cultured in serum or defined medium, reached ~80% confluence on average within 21 and 7 days, respectively. The cells in defined medium maintained a rapid proliferation up to 7 passages, whereas the cells cultured in serum medium proliferated at a significantly lower rate **(Fig. 5.1A)**. Furthermore, the cells from one donor did not proliferate in the serum medium in P0. The majority of cells cultured in both mediums displayed a regular spindle-like morphology **(Fig. 5.1B)** although a number of cells grown in the serum medium were bigger in size with what appears to be stress fibers **(Fig. 5.1 B)**.

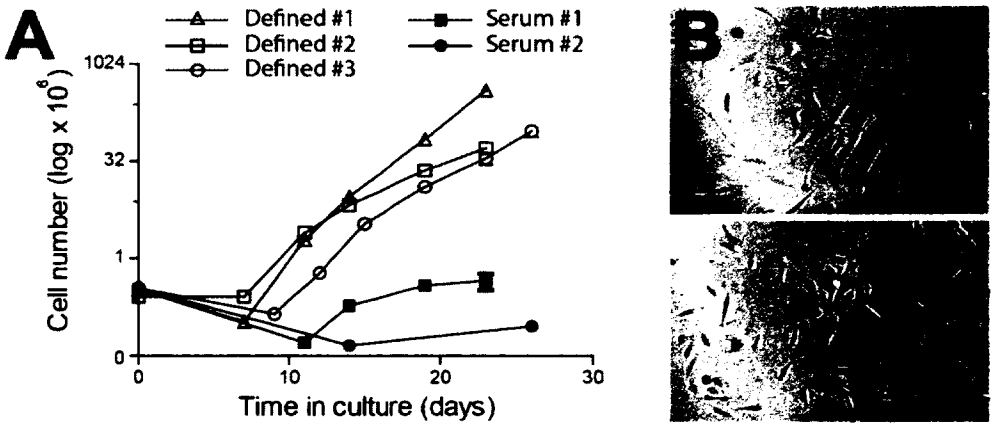


Figure 5.1: Proliferation and morphology of adherent human muscle derived cells in serum versus defined media

(A) Logarithmic cell growth population curve of adherent human skeletal muscle derived cells in serum or defined medium (n=3;M52, M63 and M38). The cells from one donor (M38) did not proliferate in serum medium. **(B)** Representative phase-contrast images of human muscle derived

cells in serum or defined medium (scale bar, 500 μm). The data are from cell lines derived from 3 different donors for serum and defined medium.

5.1.2. A sub-population of human muscle-resident cells expresses stromal progenitor markers

Adherent cells, enzymatically isolated from whole human skeletal muscle biopsies, were amplified in defined culture medium until passage 2. At this point, the cells were fractioned by FACS based on the differential expression of established mesenchymal (CD105, CD73, CD90), hematopoietic (CD34) and endothelial (CD31) cell surface markers **(Fig. 5.2A)** (Dominici *et al.* 2006). To begin, hematopoietic and endothelial cells were excluded, by $CD34^-$ and $CD31^-$ gating on viable cells. Then, an initial stromal subset was defined based on $CD105^+$ and $CD73^+$ expression, which was further separated into $CD90^-$ and $CD90^+$ cells. These subpopulations represent respectively 11±8% and 41±2% of the total viable cell population **(Fig. 5.2B)**. Following the sort, the cells were continuously cultured in defined medium. The $CD73^+CD105^+CD90^-$ cell sub-population exhibits a similar spindle-like morphology *in vitro*, in contrast to the $CD73^+CD105^+CD90^+$ cells which have an elongated shape **(Fig. 5.2C)**.. Their aspect ratio of 3.2±0.7 and 8.4±2.8 respectively support these observations **(Annex IV)**. As expected, unsorted control cells displayed a heterogeneous morphology The expression of PDGFRα (CD140a) was also evaluated by flow cytometry analysis during the sort **(Fig. 5.2D)**. In total, 58±6% of cells expressed PDGFRα. Interestingly, the majority of these cells ended up in the $CD73^+CD105^+CD90^-$ subset (93±3%), whereas the $CD73^+CD105^+CD90^+$ subset only had 17±1% expression of this mesenchymal marker **(Fig. 5.2D)**.

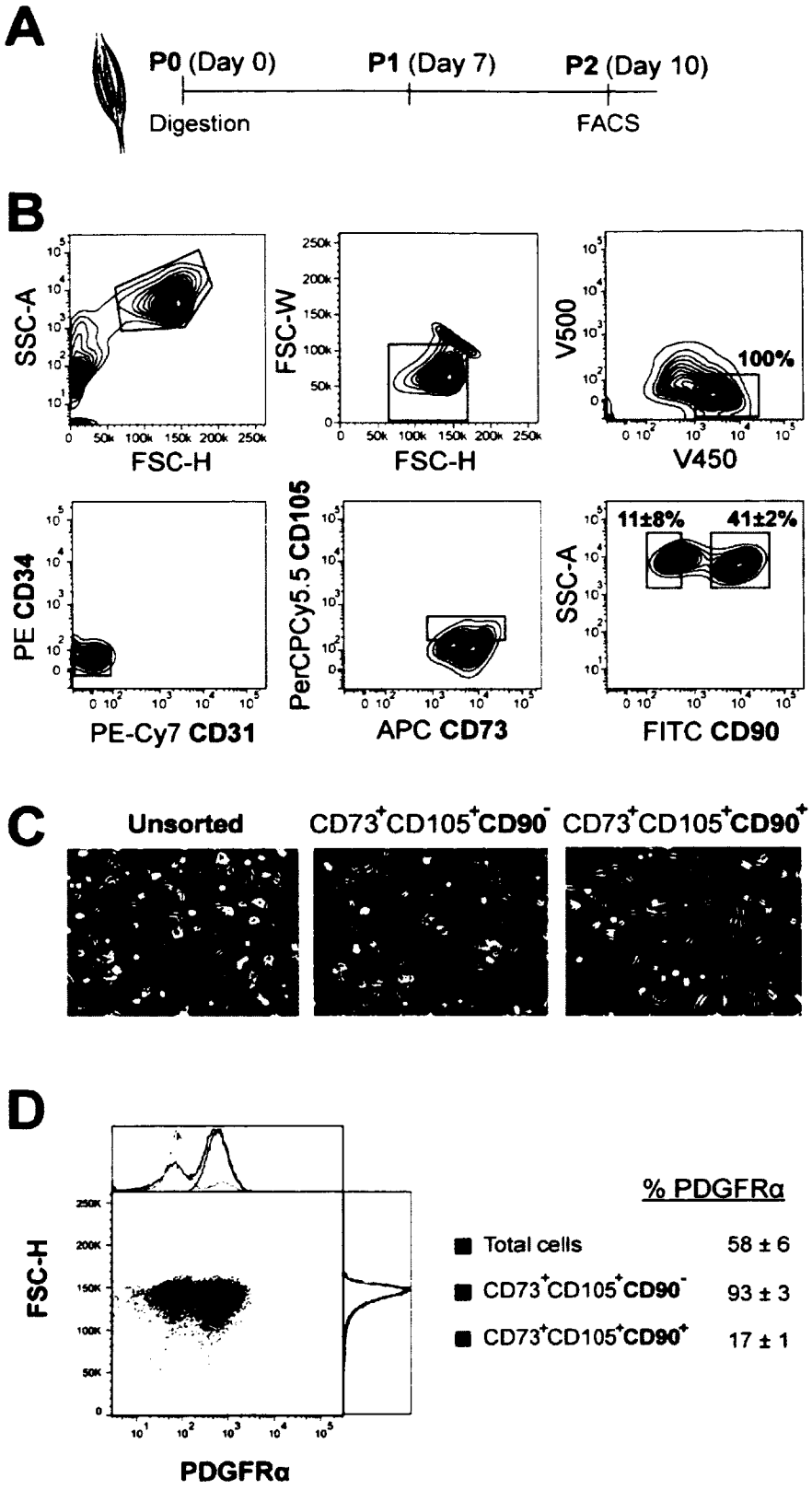

A
P0 (Day 0)
P1 (Day 7)
P2 (Day 10)
Digestion
FACS
B
SSC-A
FSC-H
FSC-W
FSC-H
V500
V450
100%
PE CD34
PE-Cy7 CD31
PerCPCy5.5 CD105
APC CD73
SSC-A
FITC CD90
11±8%
41±2%
C
Unsorted
CD73+CD105+CD90-
CD73+CD105+CD90+
D
FSC-H
PDGFRα
% PDGFRα
Total cells 58 ± 6
CD73+CD105+CD90- 93 ± 3
CD73+CD105+CD90+ 17 ± 1

Figure 5.2

Figure 5.2: Flow cytometry sorting strategy of human skeletal muscle stromal cell subpopulations

(A) Enrichment timeline of the stromal progenitor population from human skeletal muscle via FACS. **(B)** Gating strategy of representative population M38 to sort human muscle resident mesenchymal stromal cells (hmrMSCs). Cells were forward (FSC) and side-scatter (SSC) gated to exclude debris and doublets. Total live cells were gated based on calcein violet fluorescence (V450) and exclusion of the aqua dye (V500). Hematopoietic (CD34) and endothelial (CD31) cells were excluded from analysis. Two populations of $CD73^{+}CD105^{+}$ cells were sorted based on differential expression of CD90. The percentages of each cell population are shown in the panels and are expressed as the mean of six independent experiments (M37, M38, M39, M60, M63, M68). **(C)** The "$CD90^{-}$" subpopulation has a spindle-shaped fibroblast-like appearance whereas the "$CD90^{+}$" subpopulation has an elongated morphology as shown by phase contrast microscopy (scale bar, 500μm). Representative images from the M38 sample. **(D)** The distribution of PDGFRα is shown in a flow cytometry dot plot analysis. Total live cells are black, the $CD73^{+}CD105^{+}CD90^{+}$ subset is shown in grey, whereas the $CD73^{+}CD105^{+}CD90^{-}$ subset is shown in blue. The percentage of PDGFRα positive cells in each of these cell populations is expressed as a mean of two independent experiments (M58, M68).

5.2. Differentiation potential of human muscle resident mesenchymal stromal cells

5.2.1. Only $CD73^{+}CD105^{+}CD90^{-}$ cells are multipotent in vitro

After sorting, the $CD73^{+}CD105^{+}CD90^{-}$ and $CD73^{+}CD105^{+}CD90^{+}$ cell subpopulations were cultured under osteogenic, adipogenic and chondrogenic culture conditions to compare their differentiation potentials. Unsorted cells were used as a control. Alizarin red S staining revealed that only unsorted and $CD73^{+}CD105^{+}CD90^{-}$ cell subpopulations efficiently differentiated into osteoblasts, shown by the formation of mineralized nodules **(Fig. 5.3A)**. Alizarin Red S staining colored calcium deposits in red. However, $CD73^{+}CD105^{+}CD90^{+}$ cells did demonstrate minimal mineralization.

Upon adipogenic induction the $CD73^{+}CD105^{+}CD90^{-}$ cells strongly differentiated into adipocytes, adopting a spherical shape and accumulating lipid droplets, confirmed by Oil red O staining **(Fig. 5.3B)**. Isolated cells also demonstrated some adipogenic differentiation in the unsorted population, whereas no adipocytes were observed in the $CD73^{+}CD105^{+}CD90^{+}$ cell population.

Pellet cultured $CD73^{+}CD105^{+}CD90^{-}$ cells in chondrogenic medium generated chondrocytes as indicated by tissue morphology and confirmed by Alcian blue staining.

Unsorted cells display some chondrogenic capacity, while $CD73^+CD105^+CD90^+$ cells display minimal differentiation **(Fig. 5.3C)**.

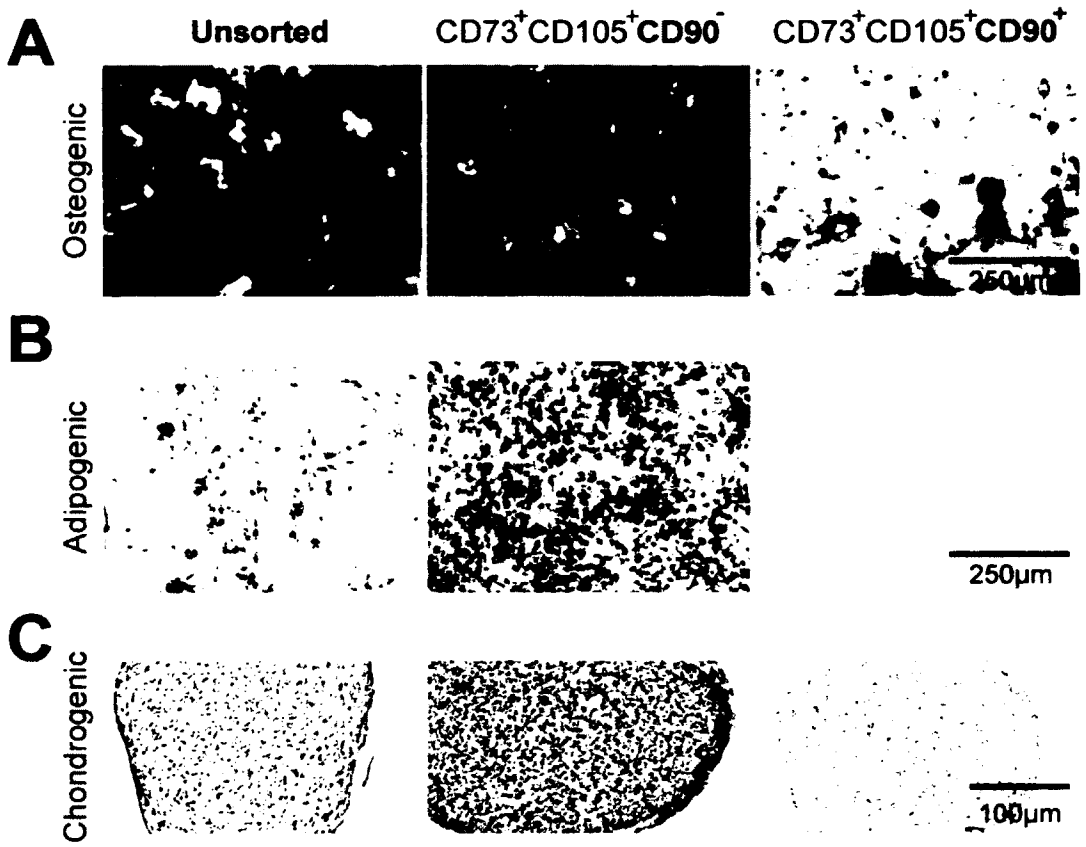


Figure 5.3: $CD73^+CD105^+CD90^-$ cells demonstrate superior osteogenic, adipogenic and chondrogenic activity *in vitro*

$CD73^+CD105^+CD90^-$ cells demonstrate superior osteogenic, adipogenic and chondrogenic activity *in vitro* as compared to unsorted and $CD73^+CD105^+CD90^+$ cells. **(A)** Mineralized, multilayered nodules in cultures grown in osteogenic medium, stained with alizarin red S (scale bar, 250µm). **(B)** Triglycerides in mature adipocytes were detected by oil red O staining (scale bar, 250µm). **(C)** Alcian blue positive cartilage in paraffin wax 5µm-sections of cell pellets grown in chondrogenic conditions (scale bar, 100µm). Representative images from sample M38 are shown. Each experiment was performed in triplicate with cells from three independent donors (M37, M38, M39 and M41).

5.2.2. Clonal progenies of $CD73^+CD105^+CD90^-$ hmrMSCs are multipotent

To address the notion that hmrMSCs arise from a common progenitor, we isolated clones from the $CD73^+CD105^+CD90^-$ cell population to determine their lineage commitment and differentiation potential. **Table 6** provides preliminary results for the frequencies of clonal progenies derived from two independent donors (M60 and M68). In total, 9 single-cell derived clonal populations were established from 576 plated wells by limiting dilution (< 1 cell/ well), representing a clonal efficiency of approximately 1.6%. Of these 9, 4 clonal progenies (~44%) differentiated into all three lineages, notably osteogenic, adipogenic and chondrogenic **(Fig 5.4)**. Three clonal progenies (~33%) displayed bipotent differentiation capabilities, differentiating towards the osteogenic and adipogenic or the adipogenic and chondrogenic lineages, whereas one clonal population (~11%) was unipotent and differentiated only towards the adipogenic lineage **(Table 6)**.

Table 6: Differentiation potential of $CD73^+CD105^+CD90$- clones

Clones[1]	n	Differentiation potential (%)
Yielded progenies	9	100%
Tripotent	4	44%
Bipotent	3	33%
Unipotent	1	11%
No differentiation	1	11%

[1] 576 hmrSCs were plated in total

The non-clonal parent cells ($CD73^+CD105^+CD90^-$) readily differentiated into all three lineages (osteogenic, adipogenic and chondrogenic). Similarly, the derived clones shown here **(Fig 5.4)** differentiated into osteocytes that produced a mineralized matrix shown by a positive alizarin red S staining, adipocytes that stained positive for Oil red O and mature chondrocytes which stain positive for Alcian blue. This preliminary clonal data suggests that human mrMSCs ($CD73^+CD105^+CD90^-$) contain cells that can differentiate into all three lineages, a defining characteristic of mesenchymal stromal cells. Interestingly, 1 of the 9 clones failed to differentiate at all, probably because these cells were already

terminally differentiated or senescent. More clonal derivations are necessary to establish a representative differentiation potential.

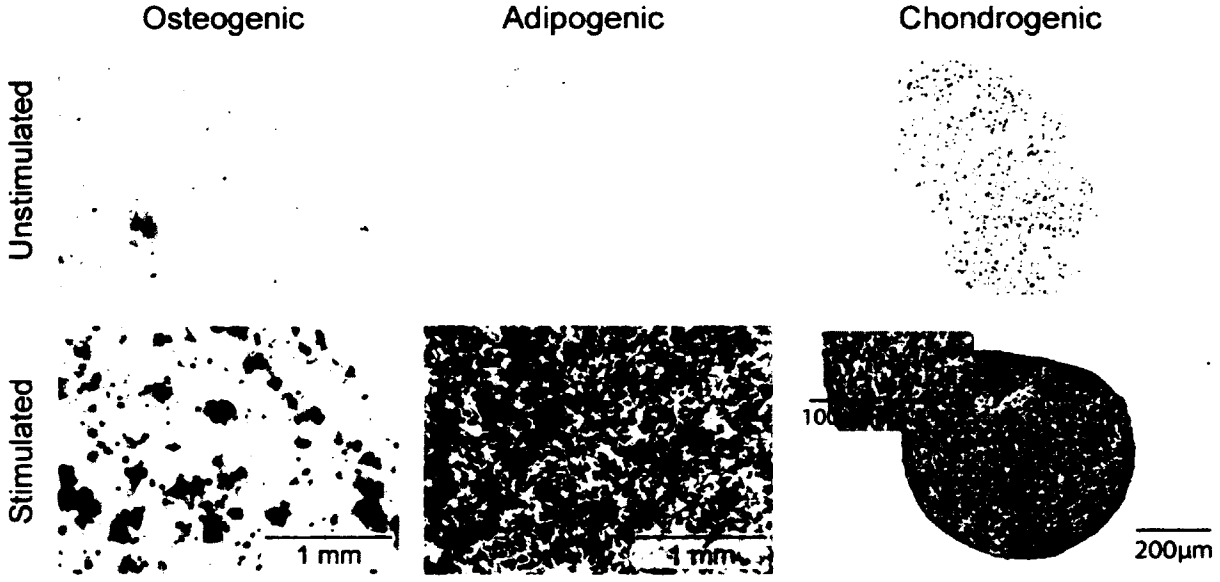


Figure 5.4: Clonal progenies of $CD73^+CD105^+CD90^-$ cells are multipotent *in vitro*

To address the heterogeneity of typical cultures of stromal cells, we established single-cell derived clones by limiting dilution of the $CD73^+CD105^+CD90^-$ subset. The differentiation of a representative clonal progeny (M60) towards the osteogenic, adipogenic and chondrogenic lineages *in vitro* shows robust differentiation towards all three lineages in comparison to the unstimulated control. Alizarin red S staining was used to detect calcium deposits (Scale bar, 1mm), oil red o staining colored lipid vacuoles (Scale bar, 1mm) and Alcian blue colored proteoglycans (Scale bar, 200µm). A higher magnification of the chondrogenic pellet shows typical cartilage lacunes with chondrocytes inside a characteristic of efficient chondrogenic differentiation (Scale bar, 100µm).

5.2.3. The effect of BMP9 on osteogenic differentiation of human muscle derived cells

We have previously shown that BMP-9 has a strong osteogenic effect on mouse muscle resident stromal cells (Leblanc *et al.* 2011). We therefore carried out a comparative study to verify the osteogenic potential of BMP9 on all three human muscle cell subpopulations. Under normal osteogenic conditions, both the unsorted and $CD73^+CD105^+CD90^-$ subpopulations differentiated into mature osteoblasts, whereas the $CD73^+CD105^+CD90^+$ subset displayed minimal mineralization, as expected (alizarin red S

staining). The addition of 2 nM of BMP9 to the complete osteogenic media or to the osteogenic media without dexamethasone decreased mineralization intensity in all three subsets, most notably in the unsorted and $CD73^+CD105^+CD90^+$ subsets **(Fig. 5.5)**.

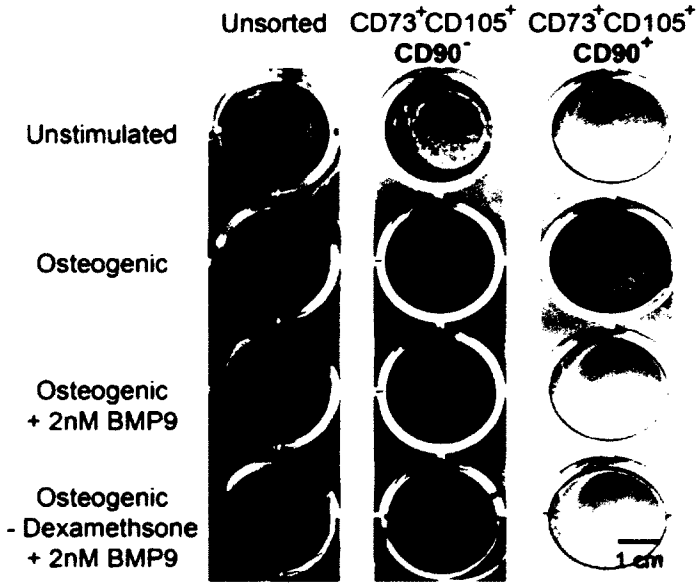


Figure 5.5: Osteogenic effect of BMP9 on human muscle resident cell subsets

Osteogenic differentiation of hmrMSC subset by BMP9. These cells were treated with control media, osteogenic media alone, or with 2 nM BMP9 or without dexamethasone and with 2nM BMP9 for 21 days. Mineralization was then observed by alizarin red S staining. Each experiment was performed in triplicate with cells from three independent donors (n=3; M37, M38 and M39). (Scale bar, 1 cm)

5.2.4. Decreased myogenic cells in the $CD73^+CD105^+CD90^-$ subset

To determine the distribution of myogenic cells following the sort, passage 3 cells of each subset were stained for the paired domain transcription factor Pax7 **(Fig. 5.6A)** which is specifically expressed by quiescent satellite cells and proliferating muscle progenitor cells (Seale *et al.* 2000). Initially, approximately 20±9% of unsorted cells expressed Pax7. After the sort, few $Pax7^+$ cells were found in the $CD73^+CD105^+CD90^-$ subset (2±2%), whereas the $CD73^+CD105^+CD90^+$ subset was significantly enriched in $Pax7^+$ cells (40±12%) **(Fig. 5.6B)**.

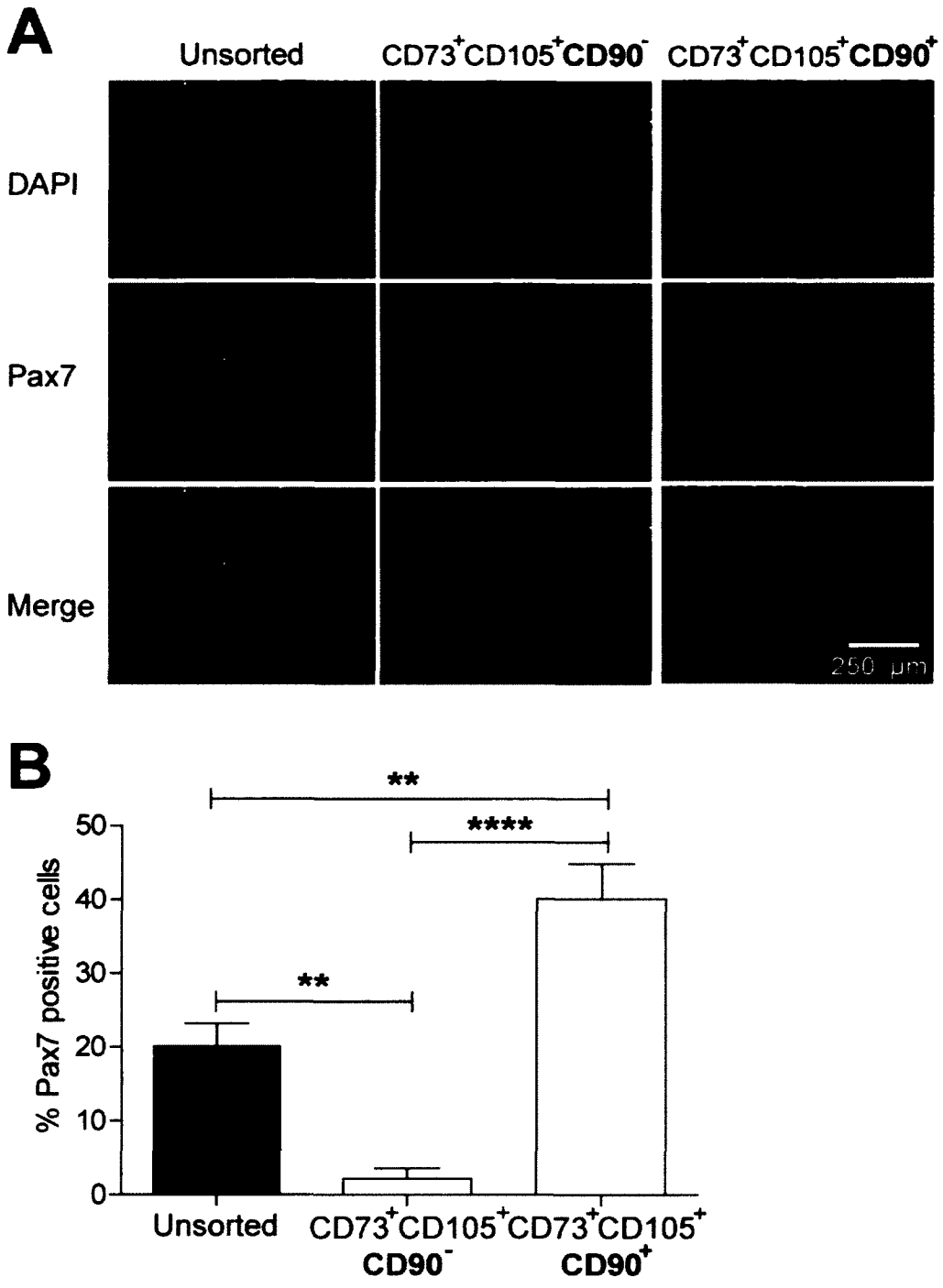


Figure 5.6: Expression of Pax7 in human muscle cell stromal subsets

(A) Representative fluorescent immunohistochemistry of $Pax7^+$ cells in human muscle stromal subsets at passage 3 (M37); Pax7 (green) and nucleus (blue) (Scale bar, 250 µm). **(B)** Quantification of $Pax7^+$ cells (expressed as a mean ± SD) of three independent experiments (n=3; M36, M37 and M38). Significant differences (****P<0.0001; **P<0.002).

5.3. Other progenitor populations present in human skeletal muscle

5.3.1. Prospective brown adipocyte progenitors in the $CD73^+CD105^+CD90^-$ subset

Schulz *et al.* identified inducible brown adipocyte progenitors residing in mouse skeletal muscle (Schulz *et al.* 2011). Furthermore, it has been previously shown that human multipotent adipose-derived stem cells (hMADS) were able to differentiate into white and brown adipocytes depending on the length of activation by ROS (Elabd *et al.* 2009).

We analyzed the expression of classical adipogenic markers such as fatty acid binding protein 4 (FABP4), adiponectin (ADIPOQ) and peroxisome proliferator activated receptor gamma (PPARγ) as well as a gene found to be highly expressed in mouse brite adipocytes: uncoupling protein 1 (UCP1). Gene expression was quantified at day 14 following either 3 days or a continuous 14 days of ROS stimulation alongside the adipogenic growth media in hmrMSCs ($CD105^+CD73^+CD90^-$). All classical adipogenic genes (FABP4, ADIPOQ and PPARγ) were significantly increased both under normal adipogenic conditions (ROS day 1 to 3) and continuous ROS stimulation up to day 14 compared to the unstimulated control **(Fig. 5.7A)**. Brite specific gene UCP1 was also significantly increased following both 3 and 14 days of ROS treatment **(Fig. 5.7A)**.

In addition, significant increase in the brown-fat specific marker UCP1 protein was observed by immunofluorescence and by western blot in hmrSCs treated with ROS (regardless of the length of treatment) **(Fig. 5.7B,C)**. UCP1 protein expression, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was again significantly increased in hmrSCs treated with ROS **(Fig. 5.7C)**.

Moreover, it has also been shown that BMP7 induced the expression of brown-fat-selective genes, notably UCP1, in a mouse primary progenitor cells (Tseng *et al.* 2008). However BMP7 did not have any effect on UCP1 expression in hmrMSCs ($CD105^+CD73^+CD90^-$) **(Annex I)**.

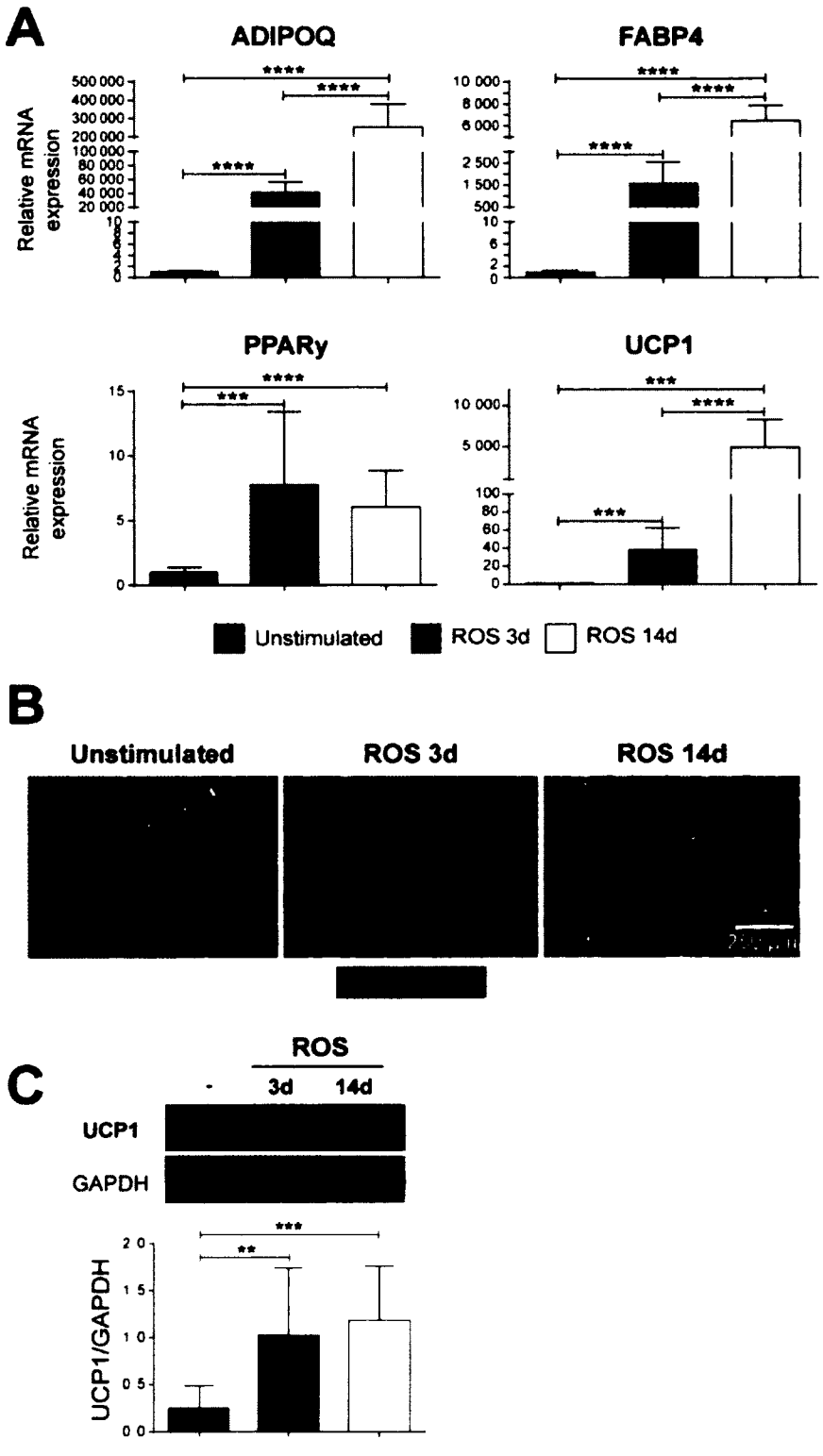

A
ADIPOQ
FABP4
PPARγ
UCP1
Relative mRNA expression
Unstimulated
ROS 3d
ROS 14d
B
Unstimulated
ROS 3d
ROS 14d
C
ROS
-
3d
14d
UCP1
GAPDH
UCP1/GAPDH

Figure 5.7

Figure 5.7: Rosiglitazone stimulates the expression of UCP1 in $CD73^{+}CD105^{+}CD90^{-}$ cells

(A) Quantitative RT-PCR of classical white and brown adipocyte genes with 0, 3 or 14 days of ROS treatment combined with standard adipogenic media. Genes were normalized to TATA-binding protein (TBP). Data are presented as a mean ± SEM (n=4; N=3; M38, M58 and M68). $*P<0.015$; $**P<0.004$; $***P<0.006$; $P<0.0001$. **(B)** Representative fluorescent immunohistochemistry of UCP1 in hmrMSCs at passage 5 (M68); UCP1 (red) and nucleus (DAPI, blue) (Scale bar, 250 µm). **(C)** Western blotting of UCP1 at 14 days following 0, 3 or 14 days of ROS stimulation and adipogenic media. Quantification of UCP1 protein expression normalized to GAPDH. Data are presented as a mean ± SEM (n=3; N=3; M38, M58 and M68). $*P<0.035$. Each experiment was performed at least three times with cells from three independent donors (M38, M58 and M68).

5.3.2. Unsorted muscle resident cells contain myofibroblastic cells.

Transforming growth factor (TGFβ1) is a potent inducer of myofibroblastic differentiation (Desmouliere *et al.* 1993), most notably through the induction of alpha-smooth muscle actin (αSMA) expression, a hallmark of myofibroblast differentiation. We analyzed αSMA expression with TGFβ1 in unsorted muscle resident cells. Few untreated cells expressed αSMA, whereas following 5 ng/ml or 25 ng/ml of TGFβ1 treatment a significant increase in αSMA^{+} cells was observed by immunofluorescence **(Fig. 5.8A)**. In a collagen gel contraction model validated in normal human dermal fibroblast (NHDF) **(Annex II)**, we found that TGFβ1 stimulation yielded significant contraction compared to the unstimulated control **(Fig. 5.8B)**. Quantitative data confirmed that 25 ng/ml of TGFβ1 stimulation yielded the most significant contraction **(Fig. 5.8C)**.

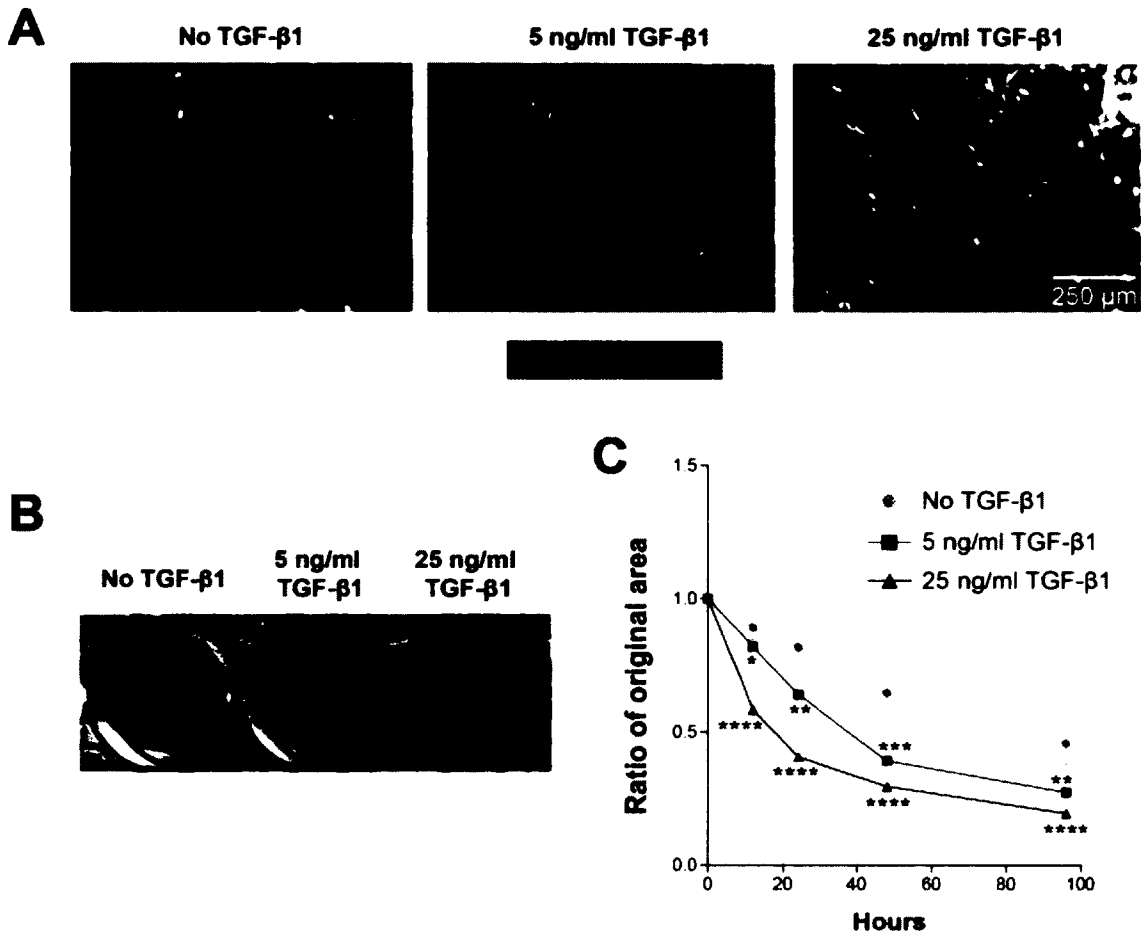


Figure 5.8: Myofibroblastic differentiation of adherent human muscle resident cells by TGFβ1

(A) Representative fluorescent immunohistochemistry of αSMA (red) and nucleus (DAPI, blue). Unsorted muscle resident cells at passage 3 expressed αSMA^{+} upon TGFβ1 treatment. Few cells expressed αSMA^{+} without TGFβ1 stimulation. **(B)** Collagen gel contraction assay showed that TGFβ1 treatment promoted significant contraction. **(C)** Quantitatively, no TGFβ1 stimulation yielded the least contraction. Stimulation of unsorted muscle resident cells by either 5 ng/ml or 25 ng/ml TGFβ1 produced significant contraction compared to the untreated cells. Data represent mean ± SEM. (n=3; N=2; M57 and M60) $*P<0.02$, $**P<0.005$, $***P<0.0008$, $****P<0.0001$.

6. DISCUSSION

Adult skeletal muscle is capable of efficient regeneration in response to a variety of insults. However when regeneration fails fibrosis, fatty degeneration and heterotopic ossification can ensue. Although the myogenic precursors responsible for regeneration have been clearly elucidated, the identity of the cells responsible for regenerative disorders such as fibrosis, fatty degeneration and heterotopic ossification in human skeletal muscle remain controversial. Identifying these cells in humans would help better understand their role in these pathologies and enable the development of treatments. Furthermore muscle tissue represents an abundant, accessible, and replenishable source of adult stem cells for cell-based tissue engineering. A population of cells isolated from muscle exhibits both multipotentiality and self-renewal capabilities.

Currently, the study of human muscle-resident mesenchymal stromal cells (hmrMSCs) is complicated by the lack of knowledge of specific markers, their relatively low abundance, their heterogeneity and the lack of a culture system that supports their multi-lineage differentiation. In this thesis, we have described an isolation technique based on the expression of specific mesenchymal stromal cell surface markers that allow the enrichment of a population of multipotent stromal progenitors with multilineage differentiation potential towards osteogenic, adipogenic and chondrogenic lineages *in vitro*. In addition, we have identified culture conditions that promote their efficient expansion.

6.1. Defined culture conditions are suitable for human muscle-derived MSC expansion

Culture conditions represent a key factor in the successful proliferation and maintenance of undifferentiated progenitor populations. Currently, there are numerous culture methods for MSCs, making standardization difficult since it has been shown that human MSC proliferation is highly dependent on growth media (Apel *et al.* 2009). As animal-derived serum is used all cases, this is less than favourable due to the dramatic influence that the serum lot may have on MSC behaviour at different levels (proliferation,

differentiation and gene expression) (Shahdadfar *et al.* 2005) and, importantly, increasing inter-laboratory variability. Furthermore, in the context of future clinical applications involving cultured human MSCs, regulatory agencies require higher safety standards for clinical-grade preparations, for safety as well as for immunologic reasons, in order to eliminate the possibility of responses against the serum components in immune competent recipients and the transfer of potential pathogens (ex. prions) (Horwitz *et al.* 2002).

Our results show that cultures of hmrMSCs can be initiated and maintained in serum-free, defined culture conditions **(Fig 5.1A)**. This is an important contribution to future clinical trials involving expanded hmrMSCs. Moreover, the proliferation rate was superior to standard serum culture conditions **(Fig 5.1A)**. Wherever time is limiting for MSC generation, this can be used to speed up the clinical-scale culture of these cells. In this context, defined medium may even reduce costs and the time needed for expansion of cells.

Typically, human MSCs isolated and expanded in classic serum-containing media are mostly spindle-shaped (or fusiform). The morphology (and size) of human MSCs may also be dependent upon culture conditions (ex. growth media and culture surface) (Qian and Saltzman 2004; Solchaga *et al.* 2005). In our experiments, cells in the serum and the defined media had a similar spindle-shaped morphology, although many cells in the serum medium appeared bigger in size **(Fig. 5.1B)**. The presence of larger cells that contained visible stress fibers suggests cell senescence (Hayflick 1965; Ksiazek 2009). This compromises hmrMSC banking possibilities as well as their multipotency.

To further ensure the safety and suitability of human muscle-derived cells for clinical applications, careful analysis of potential genetic modifications should be carried out to evaluate their tumorogenicity (Sekiya *et al.* 2002; Stolzing and Scutt 2006; Wagner *et al.* 2010), since cells might be subjected to chromosomal modifications, due to extensive cell culture and expansion (Grigorian *et al.* 2010; Nikitina *et al.* 2011; Poloni *et al.* 2011; Wang *et al.* 2012).

6.2. MSC phenotypic markers can be used to identify mrMSCs

MSCs are commonly isolated by their capacity to adhere to tissue-culture plastic and their immunological characteristics by a specific panel of markers. Because, MSCs do not express known unique phenotypic markers, the International Society for Cellular Therapy proposed minimal criteria (Dominici *et al.* 2006) for defining MSCs, notably based on their phenotype. The phenotype definition requires the expression of CD73, CD90 and CD105, together with a lack of expression of haematopoietic progenitor and endothelial cell markers (CD34), a leukocyte marker (CD45) and several others markers specific for other contaminating cell types.

We isolated a population of multipotent mesenchymal stromal progenitors from human skeletal muscle by FACS using known MSCs markers (CD73, CD105 and CD90) as well as the negative expression of haematopoietic and endothelial markers (CD34 and CD31 respectively).

In the current literature, there is a controversy regarding the expression of CD90 as a marker of mesenchymal stromal cells. Numerous articles report cell populations with high expression levels to be multipotent MSCs (Dominici *et al.* 2006; Lecourt *et al.* 2010; Chung *et al.* 2013). On the other hand, several articles suggest CD90 expression to be a marker of fibroblastic cells. One group showed that separation into $CD90^+$ and $CD90^-$ subsets in a human primary culture resulted in functionally distinct subpopulations (Koumas *et al.* 2003). Only $CD90^+$ cells were capable of myofibroblastic differentiation, whereas only $CD90^-$ cells differentiated into adipocytes, as determined by the accumulation of lipid droplets (Koumas *et al.* 2003). Also, it was shown that $CD90^+$ fibroblast-like cells in mouse skeletal muscle produce the muscle basement membrane component laminin α2 (Fukada *et al.* 2008), a role more often associated with fibroblasts than MSCs. Another group show that the elimination of CD90 expressing cells was an effective method for the elimination of contaminating fibroblasts from human primary cell cultures (Kisselbach *et al.* 2009).

Based on these results, two cellular subsets were isolated from the adherent fraction of muscle-derived cells: both, positively expressing CD73 and CD105, negatively

expressing CD34 and CD31 and distinct only in their expression of CD90 **(Fig. 5.2B)**. Up until now, both subpopulations meet the criteria used to characterize mesenchymal stromal cells in culture. They also exhibit a more uniform morphology after the sort, suggesting a more homogeneous cell population **(Fig. 5.2C)**. Interestingly, the $CD73^{+}CD105^{+}CD90^{-}$ subset was also on average 93% positive for the identified MSC marker PDGFRα **(Fig. 5.2 D)**. The co-expression of these known MSC markers in the same cellular subset may help better compare this novel muscle-resident cellular subset to others that have been identified. However, it is often difficult to compare populations identified in mice since certain markers do not have a human equivalent, for example the well identified Sca1). The use of several markers also helps fraction the unsorted muscle resident adherent fraction into different subsets with potentially different progenitor populations. On that note, it would be interesting to analyse to expression of these markers at different times, such as following the cell isolation before the cells are cultured. This would give us a better idea of the marker profile of these cells *in vivo*.

6.3. Human skeletal muscle-derived $CD73^{+}CD105^{+}CD90^{-}$ MSCs are multipotent *in vitro*

The multilineage capabilities of the unsorted and sorted human muscle-derived cells were tested using standard differentiation media **(see section 4.5)**. Only the $CD73^{+}CD105^{+}CD90^{-}$ cell subpopulation displayed multipotency *in vitro*, including osteogenesis, adipogenesis, and chondrogenesis **(Fig. 5.3A-C)**. At first glance, this result appears to go against the norm since the expression of CD90 is considered to be a marker of mesenchymal/stromal cells (Dominici *et al.* 2006). Indeed, the studies performed on human muscle-derived cells aimed to characterize the expression of stromal cell markers (CD90, CD73 and CD105) *in vitro* through several passages but without the objective to enrich specific cell subpopulations. Therefore it is the first account of the enrichment of a subpopulation expressing these markers in human skeletal muscle. Also, other studies expanded muscle-derived cells in serum based medium (Mastrogiacomo *et al.* 2005; Lecourt *et al.* 2010) which can affect the phenotype of the cells. Donor-to-donor heterogeneity can sometimes be a problem when human tissue is used. However, with our isolation and FACS-based enrichment method, reproducible multilineage differentiation

results were obtained across all the donors tested, suggesting that the chosen markers represent good targets for identifying mrMSCs in the adherent fraction of human muscle-derived cells.

Furthermore, the multilineage potential was strong up to passage 6 for all the donors tested for all three subpopulations **(Fig. 5.3A-C)** (M37, M38, M39 and M41). These donors represent 2 men and 2 women between the ages of 21 and 37 years old. This suggests that gender and age do not significantly affect the differentiation capabilities of the cells. However, our oldest donor was 52 years old. Therefore, we cannot say whether this method would be as efficient on muscle samples from an older group of donors.

The robust osteogenic and chondrogenic potential of the human skeletal muscle $CD73^{+}CD105^{+}CD90^{-}$ subpopulation has promising applications in cell-based therapies. Segmental bone loss resulting from traumatic injuries, surgical tumor removal, and reconstructive operations leave a void that can be filled by using bone graft substitutes. Currently available treatments, including autologous or allogenic bone grafting, allografts supplemented with demineralized bone matrix or osteogenic protein, and vascularized bone graft, are limited. The biological failure of engraftment is common and is probably related to the reduced osteogenic capacity of the donor bone-forming cells (Wu *et al.* 2010).

On the basis of the *in vitro* observations that muscle derived MSCs can differentiate into osteoblast, many attempts have been made to use expanded MSCs for *in vivo* bone tissue repair. In various animal models, the transplantation of muscle-derived MSCs engineered to secrete osteogenic protein leads to their differentiation into the osteogenic lineage and the accelerated healing of a bone defect (Lee *et al.* 2000; Peng *et al.* 2002). Particularly promising for orthopaedic applications, especially for bone formation, is the use of natural or synthetic biomaterials as carriers for muscle MSC delivery. Muscle MSCs have been seeded on ECM scaffolds and then implanted *in vivo* into rat, subsequently participating in bone formation (Sun *et al.* 2005). Therefore hmrMSCs are a promising cell source for cellular bone repair, particularly in delayed and non-union fractures.

On the flip side, this hmrMSC subpopulation may also represent the cellular component of HO. The ability to enrich these cells in culture may help us understand the factors and environmental cues that push these cells towards the osteogenic fate. Therefore, these cells represent an important tool in better understanding such regenerative disorders. For example, hmrMSCs represent a more appropriate tool for the screening of putative HO inductors than murine-derived stem cells or human immortalized cell lines.

The chondrogenic capacity of the human muscle $CD73^{+}CD105^{+}CD90^{-}$ cell subpopulation also has promising potential, seeing as articular cartilage has a limited capacity to heal itself, most likely due to its limited vascular supply. Currently, treatments for cartilage injury include abrasion arthroplasty, microfracture, autologous chondrocyte implantation, and meniscal or osteochondral allografts (Minas and Nehrer 1997; Komarek *et al.* 2010). However, none of these treatments fully restores the articular cartilage defect. The chondrogenic differentiation potential of muscle derived MSCs after *in vitro* culture and *in vivo* transplantation has been extensively studied (Kuroda *et al.* 2006; Claros *et al.* 2008). Adachi *et al* reported the comparable healing of cartilage defects treated with collagen gel containing either muscle derived cells or chondrocytes; this suggests that skeletal muscle contains cells that can aid cartilage repair (Adachi *et al.* 2002).

Hence, muscle derived MSCs may represent a good alternative to primary bone marrow MSCs for use in tissue engineering applications, because of their availability and the relative ease of muscle cell isolation (Qu-Petersen *et al.* 2002). Importantly, unlike human bone marrow MSCs, hmrMSCs can be easily and safely harvested in large quantities with minimal morbidity. Also, the low concentration of MSCs in the bone marrow requires the use of specialized equipment to concentrate the MSCs and may still yield too few cells (Muschler *et al.* 2005).

6.4. Multipotent clonogenic assays with $CD73^{+}CD105^{+}CD90^{-}$ cells

The hmrMSC subpopulation ($CD73^{+}CD105^{+}CD90^{-}$) displays robust multipotent capacity after enrichment. Nevertheless, the osteogenic, chondrogenic and adipogenic potentials have to be fully examined at the clonal level. A clonogenic assay will further

characterize the stem cell properties of this human skeletal muscle cell subpopulation and eliminate the possibility that the different cell fates observed stem from a heterogeneous mixture of different precursor cells. Therefore, $CD73^{+}CD105^{+}CD90^{-}$ cell clones were obtained by limiting dilution. Of the 576 plated wells, 9 single-cell derived clonal populations were established, representing a little over 1% yield **(Table 5)** most likely due to the stress clonal culture puts on primary cells. This represents a clonal efficiency similar to that observed in other studies using human primary cells (Zheng *et al.* 2013). For example, in another study on human skeletal muscle-derived cells the average cloning efficiency was 1.04%, representing 6 clones derived from 576 single-cell seeded wells (Zheng *et al.* 2013). The results from our clonogenic assay showed that 4 out of 9 $CD73^{+}CD105^{+}CD90^{-}$ clonal progenies possess strong osteogenic, adipogenic and chondrogenic potential **(Fig. 5.4)**. This experiment confirms our hypothesis that the $CD73^{+}CD105^{+}CD90^{-}$ subset contains cells capable of differentiating towards all three lineages.

6.5. BMP9 stimulation hinders osteogenic differentiation in human muscle-derived cells

As members of the TGFβ superfamily, BMPs play a critical role in skeletal development, bone formation, cellular differentiation, and stem cell biology (Attisano and Wrana 2002; Shi and Massague 2003; Luu *et al.* 2007). BMP9 is among the most osteogenic BMPs, promoting osteoblastic differentiation of MSCs both *in vitro* and *in vivo* (Kang *et al.* 2004; Luu *et al.* 2007). Our group has previously shown that BMP9 has a strong osteoinductive influence on mouse mrSCs by increasing their AlkP activity and bone-specific marker expression (Leblanc *et al.* 2011). We therefore wanted to see if BMP9 would have the same osteoinductive effect on hmrMSCs. However, unlike in the mouse model, BMP9 appeared to reduce the osteogenic ability of hmrMSCs **(Fig 5.5)**. Several characteristics of BMPs can explain this unexpected result. Perhaps the concentration of BMP9 used *in vitro* was inappropriate, either insufficient or too strong. It is a common property of members of the TGFβ superfamily to express a bell-shaped concentration response curve (M *et al.* 1996).

Furthermore, in our mouse model, BMP9 induced HO *in vivo* only in damaged muscle, compared to BMP2 (Leblanc *et al.* 2011), suggesting that perhaps another signal is necessary to induce the osteogenic fate through BMP9 signalling. Also, HO development follows an endochondral ossification process (Shore and Kaplan 2010). Perhaps, BMP9 may be a more potent inducer of chondrogenic differentiation, which latter develops into osteogenic cells. In fact, BMP9 promotes adipogenesis (Kang *et al.* 2009) and also up-regulates Sox9 expression to induce chondrogenic differentiation (Majumdar *et al.* 2001; Blunk *et al.* 2003). Overall, BMP9 has far-reaching effects beyond osteogenesis. It would be interesting to study the effect of BMP9 on human mrMSCs in another differentiation pathway than the osteogenic one.

6.6. Prospective brown adipocyte progenitors in the $CD73^+CD105^+CD90^-$ human skeletal muscle subset

Recently, brown-fat progenitors have been identified in the skeletal muscle and white fat of humans (Crisan *et al.* 2008; Elabd *et al.* 2009; Schulz *et al.* 2011), providing the potential for increasing the oxidative capacity of these tissues by targeting these endogenous precursor cells to differentiate *in vivo* into energy-dissipating brown adipocytes. Furthermore, brown-like adipocytes are found in white fat depots upon physiological or pharmacological stimulation and there is evidence that white adipocytes can be converted to brown-like fat cells (Tiraby *et al.* 2003; Cinti 2009). We have shown that hmrMSCs ($CD73^+CD105^+CD90^-$) are able to undergo efficient white adipocyte differentiation **(Fig 5.3B) whereas the other cellular subsets (unsorted and $CD73^+CD105^+CD90^+$) had very little to no adipogenic capabilities**; we then undertook a comprehensive analysis of the brown adipocyte potential of the already adipogenic $CD73^+CD105^+CD90^-$ precursor cells. A recent study described mouse "brite" adipocytes, thermogenic brown-like adipocytes, obtained upon chronic PPARγ activation of primary cultures of white adipocyte precursors (Petrovic *et al.* 2010). Another study in human adipose-derived cells showed that cells were able to differentiate into white and brown adipocytes depending on the length of activation of PPARγ by ROS (Elabd *et al.* 2009). Our observations show that hmrMSCs can differentiate into brite adipocytes following continuous ROS treatment. Following a prolonged stimulation of hmrMSCs, we denoted an

increased expression of classical adipogenic genes such as FAB4, ADIPOQ and PPARγ **(Fig 5.7A)**, as well as the brown adipocyte marker UCP1 **(Fig 5.7A)**. UCP1 expression was also increased at the protein level, although regardless of the length of treatment with ROS **(Fig. 5.7B,C)**. These results suggest that during adipogenic differentiation there is the coexistence of white adipocytes and brown adipocytes due to the simultaneous increase of markers for both cell types. Nonetheless, hmrMSCs represent a promising human cell model to analyze brown modulating signals that have been described in rodents. For example, it was observed in mouse models that BMP7 induced brown adipocyte differentiation (Tseng *et al.* 2008; Schulz *et al.* 2011). However, our preliminary results showed no effect on UCP1 expression in hmrMSCs **(Annex I)**. Similar results were observed in human multipotent adipose-derived stem cell (Pisani *et al.* 2011), highlighting the importance of human cellular models to detect the discrepancies between species. Taken together, our data suggests that differentiated brown-like human skeletal muscle-derived MSCs are representative of the so-called brite adipocytes recently described in mice (Petrovic *et al.* 2010) though significant differences are observed with regards to signaling cues favoring brown adipogenesis.

7. CONCLUSION AND PERSPECTIVES

The results of this thesis have enabled, for the first time, the identification and enrichment of a hmrMSC population based on defined culture conditions and specific MSC phenotypic markers. The isolation method we established is of great interest due to its reproducibility on a technical standpoint. Inter-laboratory inconsistency is reduced thanks to the use of a defined culture system. Furthermore, the cell surface markers chosen to isolate the population of interest seem well conserved regardless of donor-to-donor variability. Indeed, the hmrMSC ($CD73^+CD105^+CD90^-$) subpopulation displays robust multipotency *in vitro* towards the osteogenic, chondrogenic and adipogenic cell fates, and clonal progenies from this subpopulation are multipotent towards the osteogenic, adipogenic and chondrogenic lineages.

These characteristics make these cells exciting tools to help better understand regenerative disorders such as HO and fatty degeneration, which are relatively common occurrences following various skeletal muscle traumas or chronic pathologies (ex. muscular dystrophy). Understanding the mechanisms that push these progenitors towards osteogenic or adipogenic fates could help develop treatments to halt and perhaps prevent their progress. On the other hand, the multipotent potential of these cells can be harnessed to develop cellular therapies in order to treat difficult bone loss as well as cartilage defects. These are just a few of the reasons that highlight to importance of developing a culture and isolation method for human muscle-derived MSCs, and even better one that is compatible with future clinical applications.

Skeletal muscle is an excellent tissue source for progenitor cells due to its abundance and ease of access. Furthermore, skeletal muscle is a tissue with exceptional regenerative capacity, due to the presence of various progenitor populations, most notably satellite cells and mrMSCs. However, our experiments identified the presence of other progenitor populations. Brown-like adipocytes, expressing increased levels of UCP1, were identified in the hmrMSC subpopulation ($CD73^+CD105^+CD90^-$), suggesting the yet unidentified human brown adipocyte progenitors may be present in this subpopulation.

Furthermore, myofibroblastic cells expressing αSMA **(Fig. 5.8A)** and capable of collagen-gel contraction **(Fig. 5.8B)** following TGFβ1 stimulation were identified in the adherent fraction of human skeletal muscle-derived cells. These preliminary results indicate the presence of myofibroblastic progenitors, whose precise identity will be further studied in future experiments.

In summary, we have reported a reproducible isolation and enrichment method for hmrMSCs, using defined culture conditions and specific MSC phenotypic markers. The enrichment of this cell population will help us better understand the physiopathologic cell processes they participate in.

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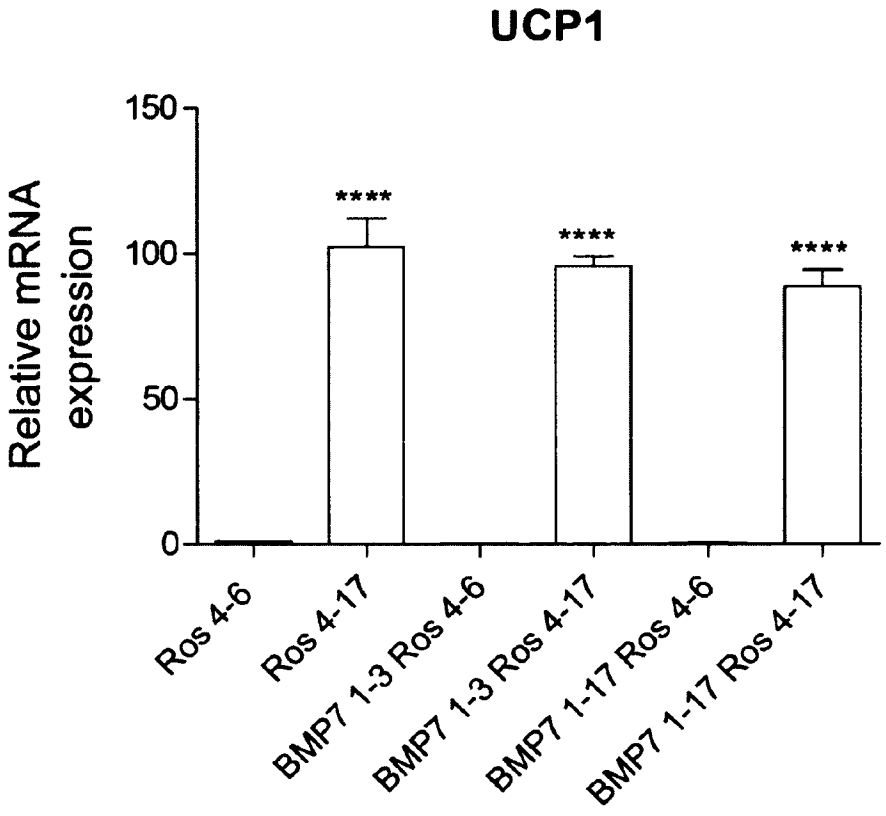
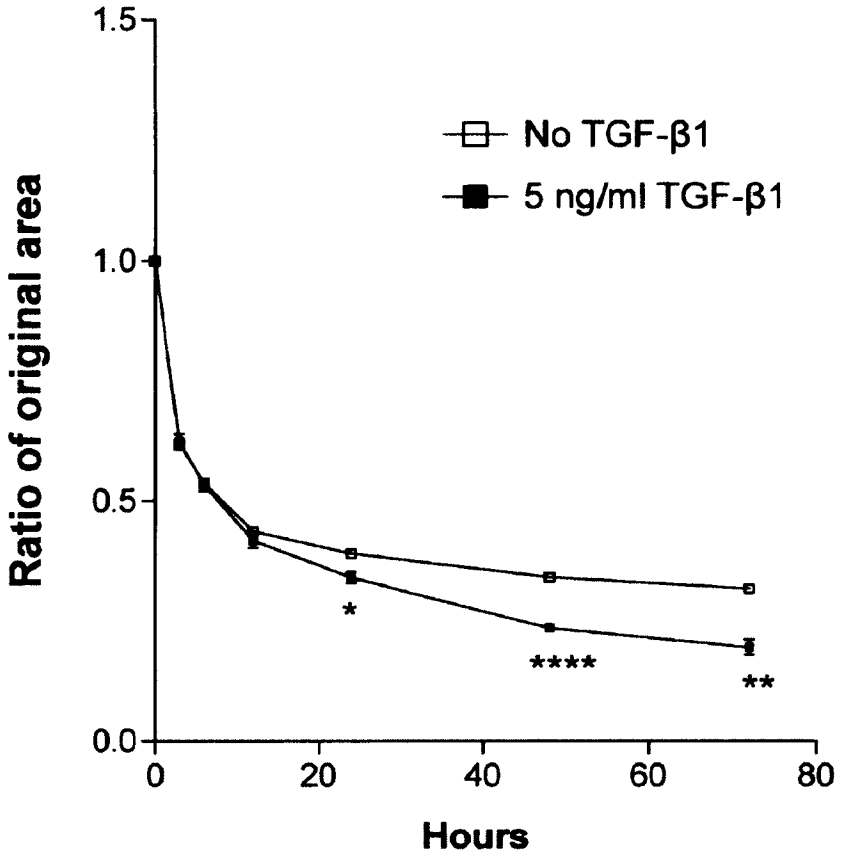
ANNEX I



Supplementary figure 1: UCP1 expression in muscle resident stromal cells with BMP7

Quantitative RT-PCR of brown fat marker UCP1 following adipogenic stimulation in combination with ROS and/or BMP7 treatment (expressed in days). Gene expressions were normalized to TATA-binding protein (TBP). Data are presented as a mean ± SEM (n=1; M58). ****P<0.0001.

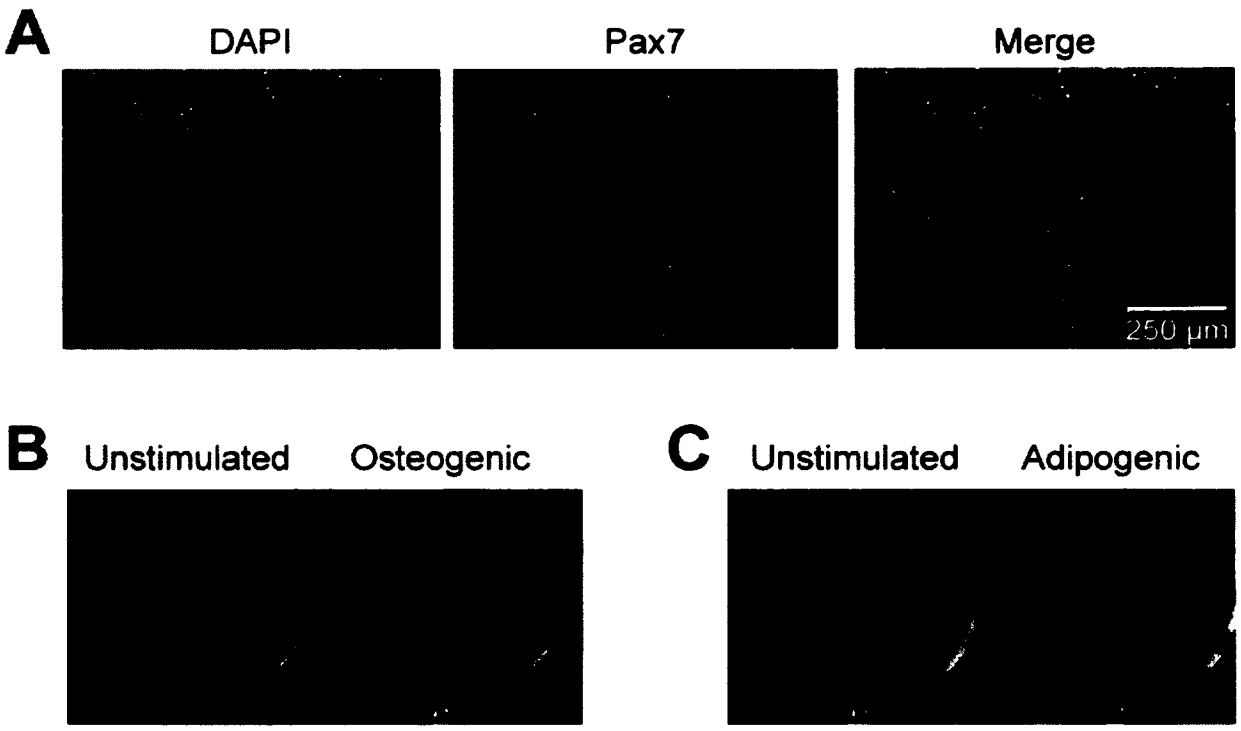
ANNEX II



Supplementary figure 2: NHDF collagen gel contraction model

Quantitatively, no TGFβ1 stimulation yielded the least contraction. Stimulation of normal human dermal fibroblast with 5 ng/ml of TGFβ1 produced significant contraction compared to the untreated cells. Data represent mean ± SEM. *P<0.02, **P<0.005, ****P<0.0001.

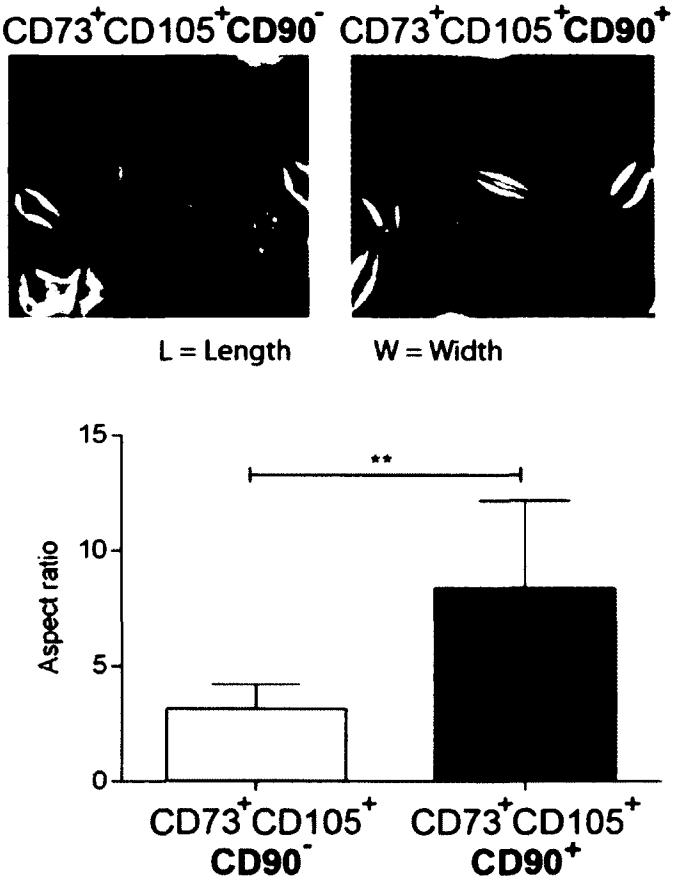
ANNEX III



Supplementary figure 3: Pax7 expression, osteogenic and adipogenic differentiation of HSMM

(A) Representative fluorescent immunohistochemistry of $Pax7^+$ cells in commercial human skeletal muscle myoblast (HSMM) cell at passage 5; Pax7 (green) and nucleus (DAPI, blue) (Scale bar, 250 µm). Under osteogenic conditions HSMM display mineralized nodules, shown by alizarin red S staining **(B)**, however no adipocytes formed under adipogenic conditions **(C)** (Scale bar, 1 mm).

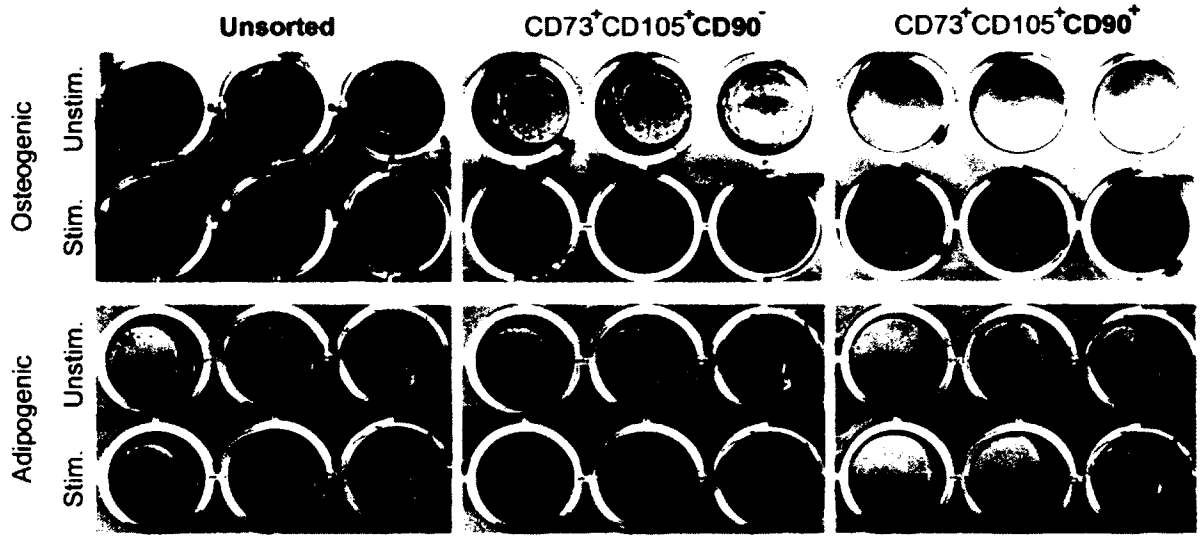
Annex IV



Supplementary figure 4: Aspect ratio comparison of $CD73^+CD105^+CD90^-$ versus $CD73^+CD105^+CD90^+$ cells

Phase contrast microscopy images of sorted populations showing representative cell morphology of the $CD73^+CD105^+CD90^-$ versus $CD73^+CD105^+CD90^+$ cell subsets (Scale bar, 125µm). Quantitative aspect ratio analysis of cell morphology (n = 50; M38). Data represent mean ± SEM. $^{**}P<0.0021$.

Annex V



Supplementary figure 5: Osteogenic and adipogenic differentiation capabilities in vitro

$CD73^+CD105^+CD90^-$ cells demonstrate superior osteogenic and adipogenic activity *in vitro* as compared to $CD73^+CD105^+CD90^+$ cells. **(A)** Mineralized, multilayered nodules in unstimulated (Unstim.) or osteogenic stimulated (Stim.) cultures, stained with alizarin red S (scale bar, 1cm). **(B)** Triglycerides in mature adipocytes were detected by oil red O staining (scale bar, 250µm). Images from the triplicate with sample M38 are shown here. Each experiment was performed in triplicate with cells from four independent donors (M37, M38, M39 and M41).